The expression of the cytokines interleukin 1beta, transforming growth factor beta and the matrix protein osteopontin by human bone cells

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DEDICATION

I dedicate this thesis to my father who always encouraged me to do my best.

SUMMARY

Two cytokines, transforming growth factor beta (TGFβ) and interleukin 1 (IL-1) have been implicated to have major roles in bone remodelling. A wealth of data has been published on the osteotropic properties of these two cytokines. It is proposed that the synthesis of cytokines in the bone microenvironment may control local remodelling. Whilst the expression of these two cytokines by osteoblasts from different animal species has been demonstrated, it has become clear that their modulation is a highly species specific phenomenon.

This study was therefore undertaken to characterise the expression of these factors in human bone. Initially the expression of TGFβ and IL-1 by human osteoblast-like cells was studied *in vitro*. The effects of various osteotropic agents on the expression of these cytokines were then monitored. In addition, the occurrence of cell surface receptors for these two cytokines was investigated. Once the expression of these factors had been established *in vitro*, the project was extended to characterise cytokine expression *in situ* using sections of human bone. There is also some evidence that matrix components may also have a role in local bone remodelling, therefore the expression of an important bone matrix protein osteopontin was also investigated.

The expression of both cytokines by human bone cells was shown to be regulated in a distinct manner. TGFβ mRNA expression was regulated by the systemic hormones, whilst IL-1β mRNA expression was modulated by cytokines and "inflammatory" agents. The idea that these factors may control local remodelling was further supported by the finding that both TGFβ and IL-1β receptors were present on the cell surface of human osteoblasts. In addition, the expression of both cytokines was detected *in situ*.

Osteopontin mRNA expression was detected in a variety of cell types in the bone tissue. The finding that osteoclasts express mRNA for osteopontin was very novel, questioning the purely resorptive nature of this cell.

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I.3 Control of bone remodelling

CHAPTER 1: INTRODUCTION

I.1 BONE AND CARTILAGE

1(i) Introduction

Bone and cartilage are two specialized connective tissues which together make up the skeletal system. There are three main functions of these tissues; firstly to give a physical framework for the body and provide sites of muscle attachment for movement, secondly to protect various internal organs, and thirdly to act as a reservoir of calcium and phosphate ions. In common with all connective tissues, bone and cartilage consist of two components namely matrix and cells. The matrix is composed of collagen fibres and a ground substance rich in proteoglycans and proteins. Unlike other connective tissues these matrices are unique since they can become calcified. The mechanism of calcification is unknown but may involve the non collagenous proteins.

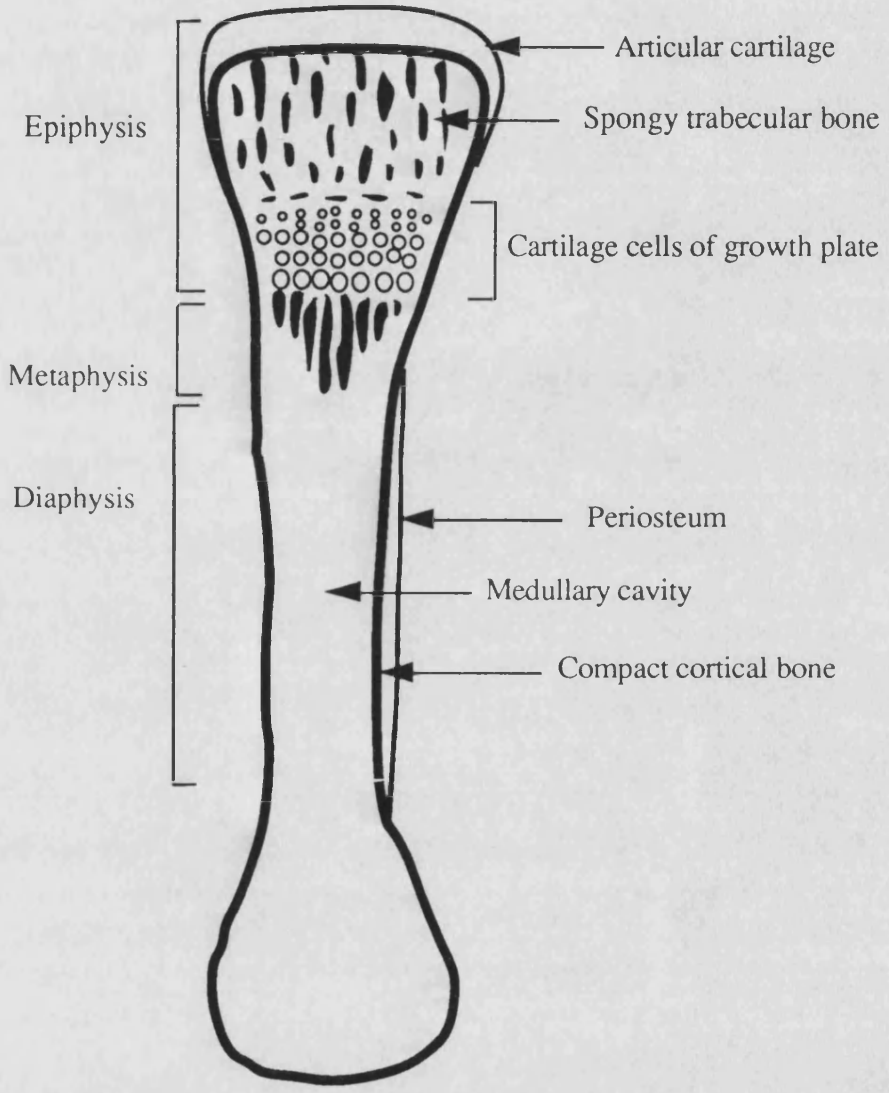
1(ii) The macroscopic organization of bone

The two types of bone in the skeleton are categorized according to their appearance; flat bones (skull bones, scapula, and mandible), and long bones (tibia, femur and humerus).

The general structure of long bones consists of three parts, the shaft of bone or diaphysis, the intermediate area the metaphysis, and the enlarged ends of bone the epiphyses (see figure I.1). During growth the epiphysis and the metaphysis are separated by a layer of epiphyseal cartilage termed the growth plate. The cartilage cells in the growth plate proliferate, differentiate and eventually become calcified, giving rise to the longitudinal growth of the bone. The external part of the bone consists of hard compact tissue termed cortical bone, which encloses the medullary cavity where red bone marrow is found in the diaphysis. Towards the metaphysis and epiphysis the cortical bone is thinner, and spongy tissue termed cancellous bone is present (Lozupone and Favia, 1990). Cancellous bone (also called trabecular bone), consists

of calcified processes (trabeculae) lined with osteoblasts interspersed with bone marrow.

Figure I.1 The macroscopic structure of bone



The general structure of long bones is shown in the diagram. Redrawn from *Functional Histology*. Churchill Livingston 1979

The bone surfaces at the epiphyses are covered with articular cartilage which does not calcify, and allows interaction with other articular surfaces in the joint. The external surface of the diaphysis is covered with vascular elastic periosteal tissue, and the inner surface with endosteal tissue. The periosteal tissue consists of; an inner osteogenic layer which contains connective cells and osteoblasts, covered by an outer fibrous periosteal layer containing blood vessels and nerves.

There are structural and functional differences between cortical and cancellous bone, however they consist of the same cell types and matrix. The structural differences are largely dependent on the degree of mineralisation of the two types of bone; 80-90% of cortical bone is mineralised compared with only 15-20% of cancellous bone. Due to its superior strength the cortical bone fulfils mainly a structural and protective role. Cancellous bone with its increased surface area is important in the metabolic functions of bone.

1(iii) The microscopic organization of bone

The microscopic "scaffolding" material of bone are collagen fibres, which orientate together in a preferential direction, and are also associated with the bone matrix. The bone matrix consists of many different proteoglycans and proteins which have a very high binding capacity for calcium ions, a property which is vital for the mineralisation process. Interspersed, in an orientated manner, between the fibres and matrix are spindle shaped crystals of hydroxyapatite. The preferential orientation of the collagen fibres alternates between layers in adult bone, giving rise to a lamellar structure which is birefringent when viewed with a polarizing microscope. These lamellae are organized in a concentric manner around a central canal in the shaft of an adult human long bone, and form numerous units of bone structure called haversian systems. The lamellae arise from layers of osteoblasts that become embedded in bone matrix and form "rings" of osteocytes separated by bone matrix. Long thin processes arise from

the osteocytes and are arranged in a pattern with respect to the haversian canal with which they connect. In addition to the concentric lamellae that make up the haversian system, other lamellae occur between systems and around the circumference of systems (see figure I.2).

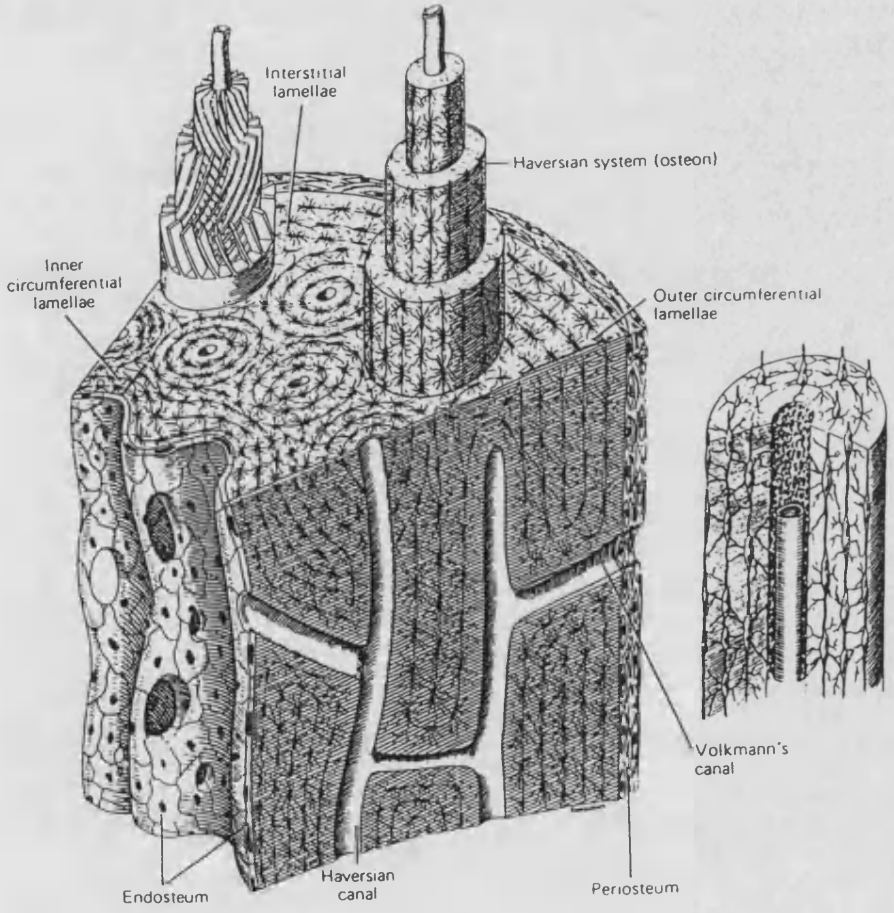


Figure I.2. Schematic drawing of the wall of a long bone diaphysis. Haversian systems, and inner and outer circumferential lamellae are evident. The protruding Haversian system on the left shows the orientation of collagen fibres in each lamella. At the right is a haversian system showing lamellae, a central blood capillary, and many osteocytes with their processes. (From *Functional Histology*. Churchill Livingston 1979)

I.1 BONE MATRIX COMPONENTS

Bone matrix is a highly complex network of over two hundred different proteins and proteoglycans. It is obviously vital as a ground substance and contributes to the microenvironment of the bone cells. Much research has been undertaken to elucidate the components of bone matrix. This research however has been hampered in the past for several reasons; initial attempts to isolate proteins from bone under strong denaturing conditions resulted in the loss of many proteins, also proteins were isolated that had not originated from bone, but were merely contaminants from other tissues. A major contribution to the field was made by Termine et al. (1980) who developed a non enzymatic procedure to purify proteins which were intimately associated with the mineralised bone. This approach was used in conjunction with sensitive techniques for protein characterisation such as high performance liquid chromatography (HPLC) and microamino acid sequencing, and allowed proteins which were present in very small amounts to be purified. However, this type of approach did not give any information on either the cell source or the regulation of synthesis of the bone matrix proteins. *In situ* hybridization and sensitive immunochemistry techniques were used to determine which cells were responsible for matrix protein production. Additionally, cell culture model systems were developed to investigate the control of bone matrix synthesis.

1(iv) Collagen

Greater than 90% of the protein in bone consists of type I collagen. Other collagens are present but only in trace amounts including types III, V, XI, XIII and X collagen (Gehron Robey, 1989). Type I collagen is a trimeric heteropolymer consisting of two alpha 1 chains [α_1(I)] and one alpha 2 chain [α_2(I)], wound together in a triple helix

to form a collagen fibril . The collagen fibrils are stabilized by the formation of covalent cross links formed from condensation reactions between allylysine and hydroxyallysine residues.

The control of collagen synthesis is obviously a very important step in bone matrix formation, and has been investigated in cell culture experiments. The major collagen synthesizing cell type in bone matrix is the osteoblast, and collagen synthesis has been demonstrated *in vitro* by osteoblast cultures of many different animal species including human, rat, and mouse (Beresford et al.1986; Canalis, 1986; Weiner et al.1989). The expression of type I collagen by osteoblasts is regulated by certain systemic hormones and growth factors, including 1,25 D_3 (Beresford et al.1986; Rowe and Kream, 1982). Cytokines are also effective agents in modulating collagen synthesis, but often have opposing effects. Centrella demonstrated that TGFβ treatment increases type I collagen synthesis in fetal rat bone (Centrella et al.1987), but similar studies revealed that interferon γ inhibits collagen synthesis both *in vivo* in the mouse (Granstein et al.1987), and in rat calvarial organ culture (Smith et al.1987).

Analysis of the type I collagen gene has lead to a greater understanding of the control mechanisms affecting this important structural protein. Promoters of the α_1 and α_2 genes have been isolated from several different species (Chu et al.1985; Schmidt et al.1986). Both contain a CCAAT box sequence, but only α_1 contains a TATA sequence. Putative enhancer binding sequences have also been identified, and are thought to be important in the control of collagen transcription. For example, it is postulated that TGFβ upregulation of collagen mRNA, is achieved through NF-1 binding to an enhancer region adjacent to the CCAAT region of the α_2 promoter (Rossi et al.1988). The first intron of the α_1 gene contains a cis acting sequence which has also been shown to increase promoter activity. It is interesting to note that the MOV-13 mouse lethal mutation, which is characterized by aberrant collagen production, is also localized to this enhancer region (Harbers et al.1984).

Since two α_1 polypeptides are required for each α_2 polypeptide to form a collagen molecule, it would be expected that the expression of the two subunits would be coordinated. This is achieved at the transcriptional level , as it has been shown that the α_1 gene is twice as transcriptionally active as the α_2 gene (deWet et al.1983). Cell culture experiments and cell free translation systems have shown that collagen expression is also regulated by a negative feedback loop (Schlimberger et al.1988), where N terminal collagen polypeptides inhibit collagen synthesis. The mechanism of this feedback is unknown but is post transcriptional, and may involve altering procollagen mRNA stability, or reducing translational efficiency.

1(v) Osteonectin

Osteonectin is a major constituent of developing bone (Fisher et al.1987), however the proportion of osteonectin in the matrix varies according to both developmental stage and species (Malaval et al.1987). In bovine matrix less osteonectin (only 40% of the maximum) is found in immature woven bone; the majority is incorporated into mature lamellar bone (Conn and Termine, 1985). Osteonectin is an acidic glycoprotein which binds both calcium ions and hydroxyapatite crystals with collagen fibres. It is thought to link various components of the mineralised matrix together (Termine et al.1981). The degree of glycosylation of osteonectin has been shown to be critical for collagen binding (Kelm and Mann, 1991).

The cDNA sequences of osteonectin from many species including mouse (Mason et al.1986) and human (Swaroop et al.1988) have been cloned, and show a very high degree of homology reflecting the evolutionary importance of this protein. Human genomic clones of osteonectin reveal a classical gene organization of 10 exons. With each exon encoding a distinct structural protein domain (Swaroop et al.1988).

Despite its name, osteonectin is not a bone specific protein (Gehron Robey, 1989). In addition to osteoblasts (Yoon et al.1987), osteonectin expression has been detected in several connective tissue cells including fibroblasts, tendon cells, and some types of

chondrocytes (Jundt et al.1987; Wasi et al.1984). Nomura screened all the major tissues of mouse and detected osteonectin transcripts in all the tissues except liver (Nomura et al.1988). *In situ* hybridization studies of fetal mouse and human bone, show that osteoblasts in the periosteal tissue and hypertrophic cartilage region express very high levels of osteonectin mRNA (Nomura et al.1988). Osteonectin has also been implicated in having a developmental role, as it is expressed in high amounts in developing adrenal glands, testes and ovaries (Holland et al.1987).

1(vi) Osteocalcin

Osteocalcin was one of the first described non collagenous bone proteins. It was originally named Bone Gla protein, by virtue of the fact that it contains gamma carboxyglutamic acid (gla) residues (Price, 1987). Osteocalcin is a comparatively small protein with a molecular weight of 5300, and has been shown to have a moderate affinity for calcium ions. The binding of these ions is thought to occur via two carboxyglutamic acid residues which are closely positioned together in the protein. The primary amino acid structure has been determined for osteocalcin by both amino acid sequencing (Poser et al.1980) and a predicted sequence from cDNA and genomic clones (Celests et al.1986). The gene is located on chromosome 1 in humans and contains 4 exons (Puchacz et al.1989). Exon 1 contains a 26 amino acid signal peptide, which is adjoined to a propeptide sequence coded by exon 2. The carboxyglutamic acid residues are contained in 2 stretches of amino acids coded by the third exon.

The tissue specificity of osteocalcin has been investigated by Northern blot analysis of RNA isolated from various tissues (Fraser and Price, 1988) and by immunolocalization (Mark et al.1987). These studies revealed that the expression of osteocalcin was very specific to mineralised tissues. Young osteoblasts and odontoblasts were the major cell types that expressed osteocalcin, and the staining

pattern suggested that osteocalcin was deposited in the bone just prior to mineralisation.

Several primary osteoblast cultures and osteosarcoma cell lines have been shown to produce osteocalcin *in vitro* (Lajeunesse et al.1990; Beresford et al.1984; Canalis and Lian, 1988). In the majority of studies the expression of osteocalcin is modulated by treatment with the systemic hormones. Both human and mouse osteoblasts increase osteocalcin synthesis following treatment with 1,25 dihydroxyvitamin D_3 (1,25 D_3) (Beresford et al.1984; Canalis and Lian, 1988). PTH treatment has also been shown to increase osteocalcin synthesis in a dose dependant manner in ROS 17/2.8 cells. The increased synthesis has been shown to be due to a concurrent increase in the steady state levels of osteocalcin mRNA (Noda et al.1988). The transcriptional rate of osteocalcin was constant but it was shown that PTH stabilized osteocalcin mRNA .

The role of osteocalcin in bone is not known, however it has been suggested that it might act to retard mineralisation until the bone matrix has reached the correct stage of maturation (Gehron Robey, 1989). This hypothesis would explain why osteocalcin is only present in large amounts in mature bone. Alternatively, since some C terminal fragments of osteocalcin are chemotactic for osteoblast-like cells (Lucas et al.1988) it may act as a recruitment factor to enhance bone formation. Osteocalcin is a vital component of bone, since bone particles devoid of osteocalcin, from warfarin treated rats, fail to be resorbed by osteoclasts (Lian et al.1986).

Osteocalcin gene structure

Osteocalcin expression appears to be regulated in a developmental stage, and cell specific manner. To determine the regulatory mechanism of this expression many studies have been undertaken to investigate the osteocalcin gene at the molecular level. It had been shown that the synthesis of osteocalcin by various osteoblast-like cells was upregulated by vitamin D_3. Therefore the first studies concentrated on characterising a vitamin D responsive element (VDRE) located upstream to the coding region.

Yoon et al. (1988) isolated the rat osteocalcin gene, and showed it was a highly conserved single copy gene. The upstream portion of the gene was cloned into a construct with a chloramphenicol acetyl transferase (CAT) reporter gene, and transfected into the rat osteoblastic cell line ROS 17/2.8. Treatment of the transfectants with 1,25 D_3 induced CAT expression by 5 - 10 fold. Various portions of the 5' upstream DNA were then deleted until a region -1035 base pairs to -871 base pairs upstream of the start codon was identified as the VDRE. A similar study was undertaken with the human osteocalcin gene and a VDRE with the classical half site arrangement was identified (Ozono et al.1990).

The gene structure of the osteocalcin gene has been elucidated from a rat genomic clone (Yoon et al.1988). It has a classical modular organization of 4 exons flanked by regulatory sequences both upstream and downstream of the coding region. The gene was shown to have 3 types of regulatory elements; an RNA polymerase II site, hormone receptor binding sites, and a 42 nucleotide sequence in the proximal region containing a highly conserved sequence termed the "osteocalcin box" (Lian et al.1989).

In contrast with type I collagen, osteocalcin is only expressed in non-proliferating osteoblasts in mineralising matrix. An elegant study by Owen et al. (1990) was undertaken to investigate how the expression of these osteoblastic developmental markers were regulated. Firstly the sequence of the upstream region of the rat osteocalcin gene was analysed. The analysis revealed that there were 2 AP-1 binding sites, which overlapped the VDRE and "osteocalcin box".

The nuclear proteins encoded by the protooncogenes c-fos and c-jun interact via a leucine zipper to form a stable heterodimer complex that together with other proteins, interacts with the AP-1 binding site. Proliferating osteoblasts were shown to contain high levels of AP-1 binding activity which dramatically changed after the down regulation of proliferation and initiation of extracellular mineralisation. Therefore a model was proposed where coordinate occupancy of AP-1, VDRE and "osteocalcin

box" sequences in proliferating osteoblasts suppressed basal and vitamin D enhanced osteocalcin gene expression. This regulation of transcription of genes associated with differentiation was termed "phenotype suppression".

1(vii) Bone sialoproteins

Two important bone matrix components are the sialoproteins, bone sialoprotein (formerly BSP II) and osteopontin (formerly BSP I). The latter will only be discussed briefly here (see chapter VII for a more extensive review). Both are glycosylated phosphoproteins and contain many sialic acid moieties (Midurat et al.1990). BSP is fairly prevalent in bone, accounting for approximately 15% of non collagenous proteins. Whilst osteopontin is present in far smaller quantities estimated at approximately 2% (Franzen and Heinegard, 1985).

A cDNA clone for BSP has been isolated from a ROS 17/2.8 library. The protein has an mRNA of 2kb which codes for 320 amino acid residues, of which glutamic acid and glycine constitute 32% (Oldberg et al.1988a). BSP is heavily glycosylated with 50% of its molecular weight in the form of O and N linked carbohydrate side chains (Fischer et al.1983). Both proteins bind tightly to hydroxyapatite in bone. They appear to form an integral part of the mineralised matrix, since they can only be extracted under denaturing conditions (Franzen and Heinegard, 1985).

BSP expression has been detected in both primary osteoblast cultures and transformed cell lines. The expression in the cell lines has been shown to be variable. UMR 106 cells produce large amounts of BSP constitutively, whilst no constitutive expression can be detected in ROS 17/2.8 cells (Midurat et al.1990). BSP expression has been detected in osteoblasts obtained from fetal rat calvariae (Yoon et al.1987). The regulation of BSP expression has been investigated using osteoblast cultures *in vitro* . 1,25 D_3 treatment has no effect on BSP synthesis, in contrast BSP transcription is stimulated by dexamethasone treatment (Oldberg et al.1989).

Oldberg suggested that BSP expression may be restricted to the differentiated osteoblast phenotype, as its synthesis follows that of alkaline phosphatase in rat osteoblasts (Oldberg et al.1989). This is supported by the *in situ* hybridization data of Bianco et (1991), who demonstrated BSP mRNA expression only in mature osteoblasts.

Despite extensive studies on these proteins in several species, their precise function in bone formation and remodelling remains to be fully elucidated. BSP can bind to collagen, therefore it may function as a link protein between hydroxyapatite and the organic bone matrix (Oldberg et al.1988a). Analysis of the primary structure of BSP reveals that it contains an RGD tripeptide sequence (Oldberg et al.1988a). This together with the fact that a BSP integrin receptor has been identified on the surface of ROS 17/2.8 cells suggests BSP may be involved in cell attachment (Oldberg et al.1988b). The BSP receptor was later found to be identical to the vitronectin receptor, and it was demonstrated that BSP promotes cell attachment *in vitro* (Oldberg et al.1988b). BSP may also be important for cell growth, since it can be shown to promote cell spreading and attachment of ROS 17/2.8 cells and bovine chondrocytes in culture (Oldberg et al.1988b; Sommarin et al.1989).

Originally BSP was thought to be highly bone specific. Northern blot analysis showed BSP mRNA to be present in large amounts in bone, but absent from other tissues (Oldberg et al.1988a). However recent research by Bianco et al. (1991) demonstrated that BSP could be immunolocalized in hypertrophic chondrocytes and placenta.

1(viii) Proteoglycans

The purification and characterisation of bone matrix components has identified 2 classes of proteoglycan in developing subperiosteal bone (Franzen and Heinegard, 1984). One large (mol wt. = 600,000) chondroitin sulphate proteoglycan, and two smaller (mol wt. = 80,000 -120,000) chondroitin sulphate proteoglycans have been

purified. The two small proteins are highly homologous (55%), although genetically distinct. It is possible that they may have arisen from the duplication of a proteoglycan gene which then evolved to give two forms. The two small proteoglycans, biglycan (formerly PGI), and decorin (formerly PGII) are not unique to bone, and have been isolated from other tissues including cartilage, skin, and tendon (Heinegard et al.1985). However it is interesting to note that whilst these proteoglycans in bone contain chondroitin sulphate moieties, those in other tissues are dermatan sulphate proteoglycans. Antisera against synthetic peptides specific for N terminal sequences of biglycan and decorin, have been used to immunolocalize these molecules in developing fetal bone (Bianco et al.1989). Both proteoglycans were immunolocalized to matrix and cells in areas of active bone formation. Biglycan alone was detected in osteogenic lacunae and canalicular spaces. The large proteoglycan was localized to loose interstitial mesenchyme where it is thought to act as a "space filler" during early bone development.

Osteoblasts from several different species including rat, mouse and human have been shown to be capable of synthesizing proteoglycans (Hunter et al.1983; Gehron Robey and Termine, 1985). Pulse chase protein studies indicate that whilst biglycan and decorin are synthesized at similar rates, the turnover of biglycan is much greater as it is modified, secreted and degraded at a faster rate than decorin (Fedarko and Gehron Robey, 1989). Osteoblasts in culture synthesize proteoglycans which are conjugated to dermatan sulphate, a form which is not bone specific. The reason for this is unknown but may reflect a dedifferentiation of osteoblast phenotype in culture. Very little data have been published on the regulation of proteoglycan synthesis by osteoblasts in culture. Treatment with 1,25 D_3 decreases the synthesis of biglycan but not decorin (Gehron Robey et al.1987a), which is interesting since 1,25 D_3 is a maturation factor for osteoblasts.

The role of the small proteoglycans in bone are unknown. It has been suggested that biglycan and decorin may diffuse to the mineralisation front ahead of other proteins

and facilitate mineralisation (Klein-Nulend et al.1981). Since proteoglycans have been shown to be associated with collagen fibres and influence both the rate of fibre growth and the diameter of fibre, it is probable that they influence the collagen scaffolding in bone (Johannson et al.1985).

Recent research has indicated that the role of proteoglycans in bone may not just be as amorphous "glue like" substances, but that they may have an important role as modulators of growth factors. Heparan sulphate proteoglycans have been shown to bind fibroblast growth factors (FGFs). The binding appears to protect the growth factors from degradation (Hausehka et al.1986). Active FGF can be liberated by proteolysis of the proteoglycan core protein or partial degradation of the heparan sulphate component. Furthermore it has been shown that the interaction of FGF with proteoglycan is essential for its function as an interaction with heparan sulphate is a prerequisite of receptor binding (Yayon et al.1991). Presumably the interaction gives rise to some conformational change of the FGF. This type of interaction with proteoglycans is not limited to FGF. Other growth factors have been shown to bind heparin, and heparan sulphate, including GM-CSF and interleukin 3 (Robert et al.1988).

The interactions described so far are mediated by the glycosaminoglycan components of the proteoglycans. Transforming growth factor beta (TGFβ) has been shown to interact in a novel manner with proteoglycans by binding to the protein core of a cell surface proteoglycan, betaglycan (Andres et al.1989). Unlike the TGFβ receptors, betaglycan does not transduce an intracellular signal but is thought to present TGFβ to the receptors. Decorin also binds TGFβ through its core protein and neutralizes its activity (Yamaguchi et al.1990). It is interesting to note that TGFβ upregulates decorin synthesis (Ruoslahti and Yamaguchi, 1991). Therefore decorin may be a vital component in a negative feedback mechanism for the regulation of TGFβ activity. Like FGF, TGFβ binding to the matrix is reversible and therefore may be an important factor in maintaining a reservoir of matrix bound growth factors.

BONE CELL TYPES

The main cell types present in bone will be discussed in the following sections. For a review of the morphological nature of bone cells see Peck and Woods (1988).

1(ix) Osteoblasts

Osteoblasts in their active state occur as a continuous layer of cells. Electron microscopic analysis shows that they contain an abundance of intracellular organelles, typical of cells vigorously engaged in protein synthesis (Jeansonne et al.1979). They form gap junctions both with their neighbours and with adjacent osteocyte processes (Doty, 1981), and contain numerous microtubules and bundles of actin filaments along the inner surfaces of their plasma membranes. Cells in a resting phase are thought to exhibit a flattened morphology, and those that are active, a cuboidal morphology. Osteoblasts are the primary cell type responsible for the synthesis of bone matrix, and are therefore highly biosynthetically active. They produce several characteristic bone matrix proteins including type I collagen, osteocalcin, osteonectin, sialoproteins and proteoglycans (Beresford et al.1984; Gehron Robey, 1989). Histochemical techniques demonstrate that osteoblasts express a wide range of enzymes including those of the citric acid cycle. Additionally they express high levels of alkaline phosphatase activity (Fritsch et al.1985) which is often used as a biochemical "marker" for these cells.

1(x) Osteocytes

Osteocytes are derived from osteoblasts which have become enclosed within lacunae in the bone matrix. Osteocytes are linked together, and to the osteoblasts on the bone surface, by thin cytoplasmic processes which extend through canaliculi permeating the bone matrix (Jeansomme et al.1979). By this route, osteocytes are able to maintain communication with each other and obtain nutrition. Osteocytes in woven bone are

plump, closely packed and have an appearance similar to osteoblasts. In contrast those in lamellar bone are widely spaced and exhibit a more flattened morphology (Parfit, 1988). Osteocytes which are positioned deep in lamellar bone are generally smaller and contain less developed endoplasmic reticulum characteristic of a less biosynthetically active cell.

The function of osteocytes remains undetermined. However since they are in contact with large areas of bone matrix, it has been assumed that they have a role in mineral exchange between the bone matrix and the circulation. It has also been postulated that osteocytes may have some role in transducing and responding to mechanical stress, since a variety of enzymes and RNA synthesis can be shown to increase in bones that have been subjected to a stress regime (Skerry et al.1989). Osteocytes are vital components of the skeleton, if the osteocytes die as a result of injury the "dead" bone is resorbed, revascularized and replaced with new bone.

1(xi) Osteoclasts

Osteoclasts are large multinucleated cells that are often attached to the bone surface. It has been established that they are the principal bone resorbing cell type, although other multinucleated cells have been implicated in bone resorption (Kahn et al.1978; Teitelbaum et al.1979). The morphological characteristics of an active osteoclast are functionally specialized, and include a ruffled border surrounded by an organelle-free actin-containing clear zone (Ryder et al.1981). Osteoclasts are rich in both mitochondria and lysosomes, and appear to contain coated pits that internalize receptor bound ligands (Kallio et al.1971). From scanning electron microscope studies on isolated osteoclasts, it has been suggested that the ruffled border of the osteoclast makes intimate contact with the bone surface and is the site of resorption (Baron et al. 1988). Pseudopodia are then thought to extend from the osteoclast and intertwine with the mineral crystals and collagen fibres. It is probable that the mineral crystals

are then phagocytosed into cytoplasmic vacuoles. The fate of the collagen fibres is not clear but probably follows an intracellular degradative pathway.

The mechanism of osteoclast mediated resorption has yet to be fully elucidated, however it seems to be an amalgamation of protease secretion together with directional ionic potential across the ruffled membrane. Osteoclasts have been shown to secrete large amounts of tartrate resistant acid phosphatase (TRAP) and other lysosomal enzymes including many acid hydrolases (Hammarstrom et al.1971). Carbonic anhydrase is also present in large amounts (Minkin and Jennings, 1972) and this enzyme may provide H^+ ions to maintain an ionic gradient across the border.

1(xii) Bone marrow

There are two types of bone marrow, red and yellow according to their microscopic appearance. Red bone marrow is the primary site of hematopoiesis, it contains an abundance of erythrocytes and blood vessels. The supporting tissue in red marrow consists of reticular fibres which support various types of progenitor cell and mature leucocytes. Red marrow is gradually replaced by yellow marrow in adult bone. Yellow marrow is less cellular and is interspersed with a large number of adipose cells. It still retains a hematopoietic potential since yellow marrow may revert to red marrow in case of severe blood loss.

2.BONE REMODELLING

2(i) Introduction

Bone is a very dynamic tissue, its continuous resorption and formation is termed remodelling. Remodelling occurs as a result of the activity of the bone cells at the surfaces of the bone, mainly the endosteal surface. Depending on the type of bone, two characteristic types of remodelling occur, Haversian remodelling and endosteal

remodelling. However the same sequence of events (termed the remodelling cycle), take place in both processes.

2(ii) Bone remodelling cycle

The bone remodelling cycle was first described by Frost (1964), from histological observations (see figure I.3). The most important feature of this process is that bone remodelling occurs at discrete sites in a well orchestrated sequence of events. In adult man 80% of trabecular and inner cortical bone, and 95% of intra cortical bone is inactive at any one time with respect to bone remodelling (Parfitt, 1983). This inactive bone is termed quiescent.

The conversion of a small area of surface bone from quiescence to activity is referred to as activation. In an adult skeleton of normal size, activation occurs approximately every 10 seconds (Parfitt, 1983). Activation occurs partly at random and partly in response to focal structural requirements. It is thought that the activation phase begins with the recruitment of mononuclear phagocyte precursors to the bone surface, which is covered by a layer of lining cells. These mononuclear phagocytic cells achieve intimate contact with the bone surface by changing shape and extending pseudopodia between the lining cells to reach the mineralised matrix surface (Chambers et al.1985). The lining cells may help this process by digesting the endosteal bone membrane and retracting to expose the bone surface. The interaction of the phagocytic cells with the bone matrix is thought to be the stimulus to promote the fusion of the mononuclear cells to form multi-nucleated osteoclast precursors.

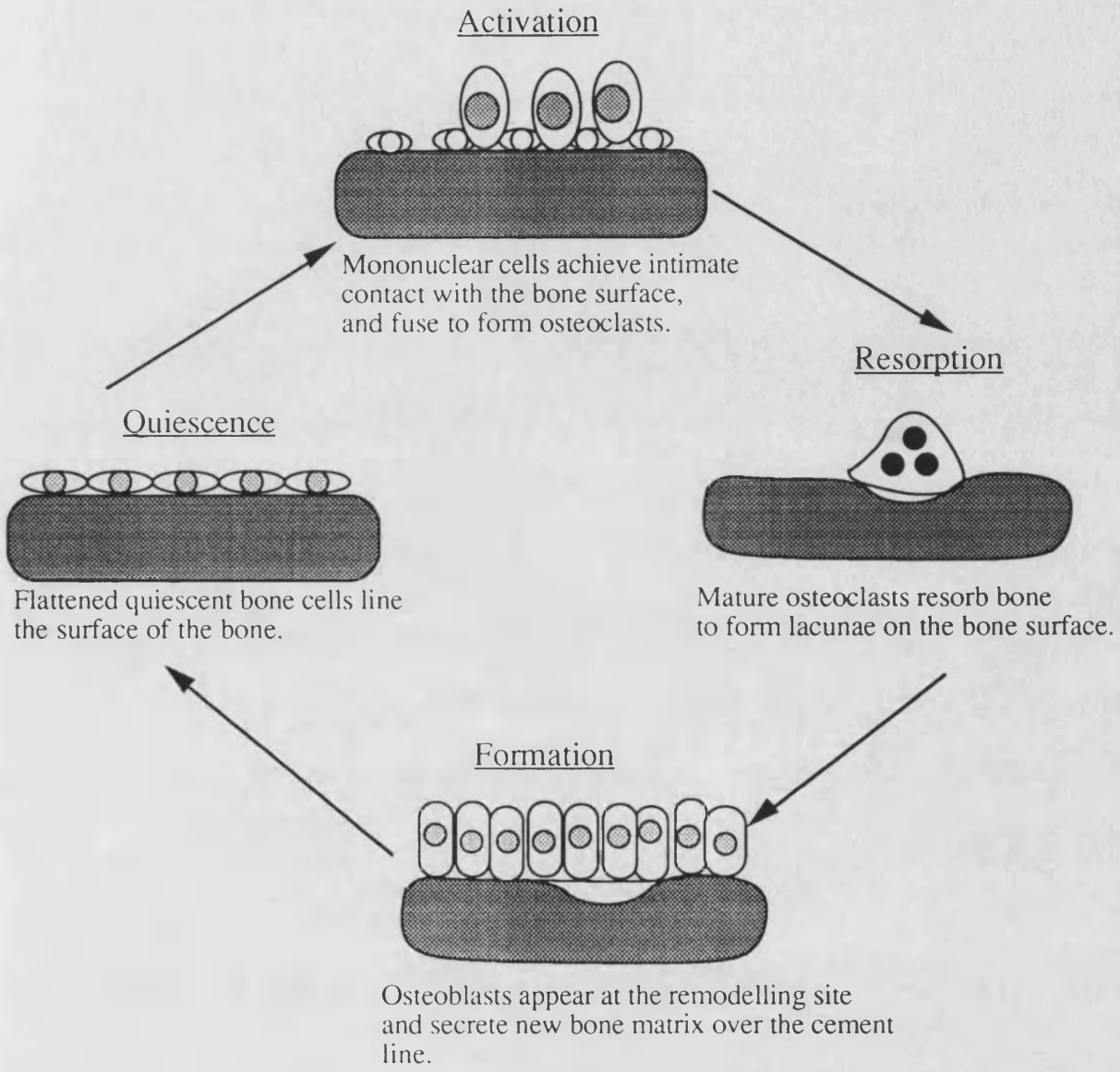
In a mature form, the osteoclasts are capable of resorbing the mineralised matrix of the bone to produce a cavity or lacuna in the trabecular surface. Scanning electron microscopic studies reveal that osteoclasts erode cavities to a mean depth of approximately 50um in trabecular bone. The resorption process is thought to take 1-3 weeks (Parfitt, 1983). The life span of canine osteoclasts has been estimated using tritiated nucleic acids and was shown to be approximately 12 days. 8% of the

osteoclasts were turned over per day (Jaworski et al.1981) which suggests a constant supply of precursors is necessary for resorption.

The next step in the cycle is the "reversal" phase during which osteoclast precursor recruitment ceases and the resorption cavity becomes populated by highly phagocytic mononuclear cells. It is thought that these mononuclear cells are intimately involved in the coupling of resorption to formation. The reversal phase is thought to be approximately 1-2 weeks in duration in the adult skeleton (Parfit, 1988). Factors which may regulate the coupling of resorption to formation, include the release of cytokines into the microenvironment of the resorption lacunae, and the bone matrix. The bone cement line deposited by these mononuclear phagocytes contains glycosaminoglycans which may be chemotactic for osteoblast precursor cells (Kahn et al.1984).

The bone formation phase follows in which osteoblasts appear at the remodelling site and secrete new matrix over the cement line. The matrix then becomes mineralised by a mechanism which remains unclear, but which may involve an affinity of certain proteoglycans for calcium ions. Normally homeostasis is achieved when the amount of bone formed by the osteoblasts matches that resorbed by osteoclasts.

Figure I.3: The bone remodelling cycle



I.2 Mechanisms of bone formation

Bone formation is a complex process since it is necessary to maintain skeletal structure in conjunction with a continuous and orderly growth of the skeleton. Two types of bone formation occur, namely intramembranous and endochondral bone formation (figure I.4 shows the histological appearance of these two types of bone formation). Both types of bone formation usually occur independently, although occasionally both occur simultaneously at different locations within a single bone. The mechanisms of both types of bone formation are described below.

2(iii) Endochondral bone formation

Tubular bones develop predominantly by endochondral bone formation (Baron, 1991). The most important feature of endochondral bone formation is that it occurs from a cartilage template. Cartilage tissue develops from clusters of mesenchymal cells which differentiate to form chondroblasts. These chondroblasts synthesize large amounts of cartilaginous matrix which is rich in type II collagen and mucopolysaccharides. The chondroblasts eventually become embedded in the matrix to form chondrocytes. In addition to developing from mesenchymal cells, chondrocytes can also divide to form other chondrocytes in the matrix. Due to this cell proliferation cartilage may expand in a widthwise manner. Developing cartilage forms a growth plate with 3 distinct zones. The first cartilage zone consists of resting cartilage cells next to the epiphyseal bone. Adjacent to the resting chondrocytes, proliferating chondrocytes form vertical columns in a proliferating zone. The hypertrophic zone where immature chondrocytes enlarge and polarize, adjoins this proliferating zone. Hypertrophic chondrocytes in this region produce alkaline phosphatase which is thought to promote the mineralisation of the cartilage. The mineralised cartilage then acts as a template for the deposition of bone in the primary

spongiosa. This region of woven bone and cartilage is remodelled at a later stage to mature lamellar bone.

2(iv) Intramembranous bone formation

Intramembranous bone formation is characterised by the growth of bone from a cluster of ossification centres without a cartilage template. At these centres, groups of mesenchymal cells differentiate directly to form preosteoblasts and osteoblasts. These cells are capable of synthesizing type I collagen and matrix proteins and produce islands of osteoid which may become mineralised. Intramembranous bone contains osteocytes that are very numerous and large in size. It has been shown by polarized light microscopy, that the collagen synthesized in this immature bone is not highly organized, but is present in a disorganized array. Calcification occurs concurrently with alkaline phosphatase expression at irregularly distributed patches. The calcified osteoid that is formed can be remodelled to give immature woven bone. Trabeculae are formed which are lined with osteoblasts which secrete more bone matrix to give rise to branches of trabeculae. At the periphery, other mesenchymal cells may differentiate to facilitate the outward growth of the bone.

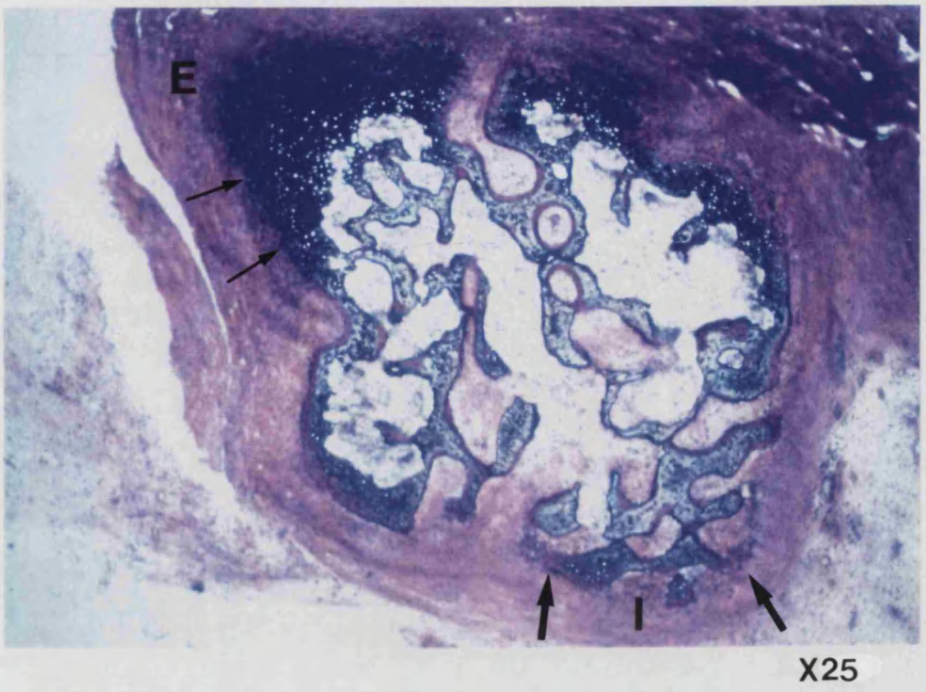


Figure I.4 : The histological appearance of both endochondral (E) and intramembranous (I) bone formation. The endochondral bone formation (small arrows) can be seen to occur on a cartilage template, whilst islands of intramembranous bone (large arrows) arise spontaneously from the newly differentiated osteoblasts.

2(v) Growth and shape modification -modelling

It is important to differentiate between the continuous turnover of bone (remodelling), and circumstances of bone growth or repair which are termed modelling. During bone remodelling the activation, resorption, formation (ARF) sequence is followed whereas during modelling this sequence is modified. For example the growth in diameter of the diaphysis of bone is a result of deposition of new membranous bone beneath the periosteum. This occurs as a result of bone formation directly after activation (AF) with no intermittent resorption. Similarly during the longitudinal growth of long bone, the metaphysis is progressively destroyed by the resorption by osteoclasts beneath the periosteum. During this process, resorption follows activation (AR) but is not followed by bone formation.

2(vi) Bone and cartilage mineralisation

It has been previously assumed that the mineralisation of the matrix was a passive diffusion based process, however recent data suggest that bone cells may control this process (for a review see Termine, 1991). A different mechanism is employed for the initial mineralisation of matrix to form woven bone or calcified cartilage than that for the synchronous calcification of matrix associated with mature bone.

The initial mineralisation of woven bone or growth plate cartilage obviously requires a large local concentration of calcium and phosphate ions to form hydroxyapatite crystals. This increase in local concentration is facilitated by small membrane bound vesicles which bud off from the cytoplasmic processes of chondrocytes and osteoblasts (Bonucci, 1971). These vesicles are thought to be deposited in the matrix during its formation and have a high affinity for calcium ions. Large amounts of phosphate ions are produced by the hydrolysis of ATP and pyrophosphate by enzymes in the membrane of the vesicles. Calcium and phosphate ions become concentrated in the vesicles and form calcium hydroxyapatite crystals. When the

vesicles membrane ruptures the crystals are released into the matrix and act as nucleators of further crystal growth. Providing the matrix is in a competent state to be mineralised, the crystals grow in clusters which eventually coalesce to completely calcify the matrix.

2(vii) The mineralisation front

The mineralisation of mature bone occurs in a progressive manner from the calcified bone to the osteoid. The lag phase between the osteoblastic synthesis of osteoid and mineralisation is termed the osteoid maturation period. During this period the matrix is modified so that mineralisation can occur. This modification is thought to involve a series of changes in matrix composition, where certain proteoglycans and phospholipids are removed, and water is excluded. During the advancement of the mineralisation front calcium binds to non collagenous proteoglycans to reduce their degree of hydration. This dehydration of the matrix in turn causes an influx in calcium from the plasma leading to increased calcification of the osteoid.

I.3.CONTROL OF BONE REMODELLING

I.3 THE SYSTEMIC GROWTH HORMONES

The following section will review the influence of systemic growth hormones on bone metabolism. For a long time is has been known that these factors are of primary importance in bone homeostasis. Many medical conditions exist where a perturbation in hormone levels leads to gross skeletal abnormalities. Examples include Vitamin D dependant rickets and hyperparathyroidism.

3(i) Vitamin D_3: regulation of Vitamin D_3 metabolism

There are many diverse effects of vitamin D_3 on skeletal tissue (for review see Deluca, 1982). The following section concentrates on the regulation of vitamin D_3 action and

its effects on cells of the osteoblastic and osteoclastic lineages. This vitamin shares many properties of a hormone. For example, target tissues such as intestine, kidney and bone contain specific high affinity receptors for vitamin D_3. Vitamin D_3 is obtained from the diet or synthesized in the skin from dehydrocholesterol in a reaction catalysed by u.v. irradiation.

Once synthesized, vitamin D_3 is conjugated to specific binding proteins and is transported via the circulation to the liver. In the liver it is hydroxylated to its derivative 25 hydroxyvitamin D_3 [25(OH)D_3]. The biologically active metabolites of vitamin D_3 are the dihydroxylated derivatives, 1,25 dihydroxyvitamin D_3 [1,25 D_3] and 24,25 dihydroxyvitamin D_3 [24,25 D_3]. These metabolites arise as a result of further hydroxylation of 25(OH)D_3 in the kidney mitochondrion, catalysed by the enzymes 1-α-hydroxylase and 24-α-hydroxylase respectively. Relative levels of 1,25 D_3 and 24,25 D_3 have been shown to depend on the endocrine status of the individual. The systemic hormone parathyroid hormone (PTH) stimulates the activity of 1-α hydroxylase when there are low levels of circulating 1,25 D_3. The over production of this hormone is avoided as there is a negative feed back loop for 1,25 D_3 which inhibits it's own synthesis by suppressing 1-α hydroxylase activity.

3(i) Molecular mechanism of 1,25 D_3 action

It is postulated that 1,25 D_3 has a mode of action similar to the steroid hormones. Circulating 1,25 D_3 is thought to pass into the cell due to its solubility in the cell membrane. Once in the cell it is thought to bind to a specific high affinity receptor. The 1,25 D_3 receptor has been biochemically characterised and is a protein of 61kDa with a very high binding affinity for 1,25 D_3 (Kd = $1x10^{-10}$) (Baker et al.1988). The steroid receptor complex is then thought to be transported to the nucleus, where a distinct receptor domain binds with a DNA vitamin D responsive element (VDRE) upstream of the target gene. Once the steroid receptor complex has bound to the 5' upstream region of the target gene, transcription is thought to be altered by interactions between

the receptor and repressors or initiation factors. The primary amino acid sequence of the 1,25 D_3 receptor shows that this protein contains many regions homologous with other steroid receptors (Evans, 1988). The DNA binding domain of the receptor contains clusters of cysteine residues that give rise to two zinc finger-like structures which are thought to wrap around the DNA.

Mutation analyses of VDRE consensus sequences have revealed an exquisite selectivity of certain sequences for steroid receptors. It seems that it is not only the sequence itself that determines whether a certain receptor will bind, but rather the spacing between DNA motifs (half sites). An elegant study by Umesono et al. (1991), demonstrated that a thyroid responsive element (TRE), could be converted to a retinoic acid responsive element (RARE) by increasing the nucleotide spacing between half sites by 1 nucleotide. Decreasing the nucleotide spacing by 1 nucleotide converted the TRE to a VDRE.

3 (i) The actions of 1,25 D_3 on osteoblasts

Table 1 shows that 1,25 D_3 receptors have been found in many osteoblast cell lines. The number of receptors present in the osteoblasts may be modulated by many factors including the state of cell confluency and corticosteroid treatment. For example Chen et al. (1983b) showed that the number of 1,25 D_3 receptors in mouse, but not rat osteoblasts, was dependent on the proliferative status of the cells. In cultures where the cells were confluent and had therefore stopped dividing, low concentrations of receptors were observed. In contrast cells which were actively dividing contained the highest receptor levels.

Proliferation studies of mouse, rat and human osteoblasts (Beresford et al.1986; Chen et al.1983a) *in vitro* have shown that 1,25 D_3 is inhibitory in a dose dependent manner (1pM-130nM). In many cell types, a decrease in proliferation is often associated with an increase in differentiation and *vice versa*. This is thought to be the case for osteoblasts, as many markers of a differentiated phenotype are induced by

1,25 D_3 such as alkaline phosphatase (Manolagas et al.1981) and osteocalcin synthesis (Beresford et al.1986). The synthesis of another bone protein, Type I collagen, by the osteoblastic cell line MC3T3-E1 is also increased following treatment by 1,25 D_3 (Kurihara et al.1986).

Table I.1 : The occurrence of 1,25 D_3 receptors on bone cells

Cell line/cell	Species	1,25 receptors	Properties	Reference
Normal lining cells	fetal rat bone	+	*	Narbeitz et al.1983
Osteoblasts	fetal rat bone	+	*	Narbeitz et al.1983
Osteoblasts precursors	fetal rat bone	+	*	Narbeitz et al.1983
Osteocytes	fetal rat bone	-	*	Narbeitz et al.1983
Osteoclasts	fetal rat bone, chick	-	*	Merke et al.1986; Narbeitz et al.1983
Normal osteoblasts	mouse (calvaria)	+	Dependent on cell density ⬇ by glucocorticoids	Chen et al.1979
Normal osteoblasts	rat (cultured)	+	⬆ by glucocorticoids	Chen et al.1983
OS-12 osteosarcoma	rat	+		Rodan et al.1987
UMR osteosarcoma	rat	+		Partridge et al.1990
MMB-1 osteoblast line	mouse	+	Dependent on cell density	Walters et al.1982
MC3T3-E1 line	mouse	+		Kurihara et al.1986
ROS17/2 line	rat	+		Dokoh et al.1984
MG-63 osteosarcoma	human	+		Franceshi et al.1985
Saos-2 osteosarcoma	human	+		Rodan et al.1987

⬆ = increase, ⬇ = decrease, + = present, - = not present.
* detected by radioligand binding to tissue sections.

3(i) The actions of 1,25 D_3 on osteoclasts

1,25 D_3 is a potent stimulator of bone resorption. Physiological doses of ($8x10^{-11}M$ - $10^{-10}M$) induce resorption in both organ cultures (Raisz et al.1972a) and in *vivo* (Reynolds et al.1973). *In vivo* administration of 1,25 D_3 to vitamin D deficient rats (Holtrop et al.1981) or mice (Tinkler et al.1981) results in an increase in osteoclast number in the bone tissue. As shown in Table I.1 mature osteoclasts do not possess 1,25 D_3 receptors, therefore this agent cannot act directly on osteoclasts. However it may promote the differentiation of stem cells and progenitors, or stimulate fusion of preosteoclasts to form mature osteoclasts. There is some experimental data to support this hypothesis, since 1,25 D_3 has been shown to increase osteoclast formation in bone marrow cultures from many species. Cultures of feline bone marrow, which spontaneously give rise to multinuclear cells, showed an increase in cell fusion within 24 hours of 1,25 D_3 treatment (Ibbotson et al.1984). However it is controversial whether the multinucleated cells that were generated in the feline bone marrow were authentic osteoclasts. Similar studies have shown that 1,25 D_3 promotes differentiation of monocyte precursor cells in marrow cultures and stimulates their fusion to form multinucleated cells (Pharoah and Heersche, 1985). The effect was species dependent as treatment of murine bone marrow cultures and fetal rat bone rudiments with 1,25 D_3, failed to stimulate fusion of mononuclear cells (Meer et al.1980).

3(ii) Oestradiol

Over 40 years ago Albright and Reifenstein noted the high incidence of osteoporosis in patients with hypogonadism (Melton and Riggs, 1988). Since then much research has been undertaken to determine the importance of sex steroids in the maintenance of skeletal integrity. Much circumstantial evidence suggests that the sex hormone status of an individual is very important. Women who are amenorrhoeic for a considerable

time, for example those suffering from anorexia nervosa show a greater incidence of osteoporosis (Rigotti et al.1984). Additionally elite athletes who are oestrogen deficient have been shown to have a lower bone mass than age matched controls (Drinkwater et al.1984). Since reasonable amounts of exercise increase bone mass, this deficiency highlights the importance of oestrogen in maintaining skeletal bone mass in females. Furthermore the administration of exogenous oestrogen to post-menopausal women retards further reduction of bone, although it does not stimulate net accumulation (Christiansen and Lindsay, 1990). Despite the general acceptance of the major role of oestrogen in reducing bone loss, there is as yet no consensus on its mode of action.

Many *in vivo* studies have also highlighted the role of oestrogen in maintaining bone mass. Histomorphometric analysis of trabecular bone in femurs of ovariectomized rats, has shown that ovariectomy causes massive reduction in trabecular bone volume. Additionally an increase in both resorption surfaces and osteoclast numbers is observed (Takano-Yamamoto and Rodan, 1990). An 8 day infusion of (10^{-9}M) 17β oestradiol largely restored trabecular bone volume and reduced the number of osteoclasts present and the resorption surfaces. This study was supported by a similar study by Wronski et al. (1988) who obtained very similar results. The data taken together from these and other studies suggests that oestrogen gives a protective effect on the maintenance of trabecular bone mass.

3(ii) Effects of oestrogen on osteoblasts

Oestradiol has been shown to affect both the proliferation and differentiation of osteoblasts *in vitro*. Two investigators have independently reported that 17β oestradiol has an inhibitory effect on the proliferation of the rat osteogenic cell line (UMR-106) (Gray et al.1987; Bankson et al.1989). In contrast oestradiol has also been shown to enhance the proliferation of primary cultures of rat calvarial bone cells (Ernst et al. 1989). Osteoblast-like cells were prepared from calvariae of new born rats and

cultured in serum-free medium containing methyl cellulose for 12 days. The osteoblasts exhibited a unique spherical morphology under these conditions, and proliferated selectively into clonally derived cell clusters. Oestrogen at concentrations of 0.1 - 1 nM was shown to enhance osteoblast proliferation by up to 68%. The effect was shown to be specific since the anti-oestrogen tamoxifen abolished the stimulatory effect of 17β oestradiol. The effect of 17β oestradiol on osteoblast proliferation, has been shown to be a species specific phenomenon. The proliferation of primary human bone cells is unaffected by oestrogen treatment over a large concentration range (Colvard et al.1989). Oestrogen seems to promote a differentiated osteoblast phenotype. It has been demonstrated to upregulate alkaline phosphatase expression (Gray et al.1987) and increase the synthesis of matrix proteins including osteocalcin, osteonectin (Glibert et al.1989), and collagen (Ernst et al.1989). Preliminary studies suggest that oestrogen may also modulate the expression of cytokines by bone cells. Recent data shows that insulin like growth factor 1 (IGFI) synthesis is increased by oestradiol treatment in human bone cells *in vitro* (Gray et al.1989).

3(ii) The oestrogen receptor

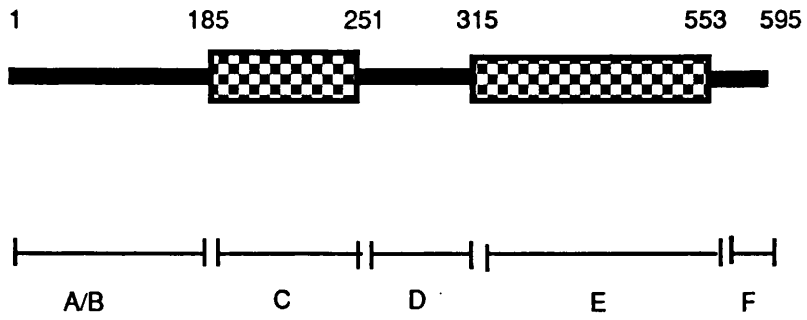
Oestrogen, in common with other steroid hormones regulates gene expression in target cells through its interaction with specific receptors. Data obtained from studies on many tissues suggest that the oestrogen free receptor is localized predominantly in the nuclear compartment where it is loosely bound. The binding of oestrogen to its receptor is then thought to convert the receptor to an active form which has the ability to bind specific promoter elements in oestrogen responsive genes.

The human oestrogen receptor was cloned by Walter et al (1985) from a cDNA library constructed from a human breast carcinoma cell line. A 6.2 kb mRNA transcript for the oestrogen receptor was identified which coded for a 595 amino acid protein of 66kDa. Sequence comparisons together with mutation analysis, have defined two conserved regions of the oestrogen receptor that correspond to important functional

domains (see figure I.5) (Gibson et al.1990). The putative DNA binding domain (C) is a highly conserved 66 amino acid region, which has the potential to form 2 DNA binding fingers via two pairs of cysteine residues. Results obtained from a chimeric receptor show that this region determines the specificity of the receptor for the transcription of the target gene. The hormone binding domain is located in the C terminal half of the receptor (E). There is also a hydrophilic region (D) which may correspond to a hinge between DNA and hormone binding domains. There has been a suggestion that the region A/B is required for activating transcription.

Hydrophobicity plots and analysis of the amino acid sequence reveal that the oestrogen receptor belongs to a steroid receptor superfamily. This family includes the receptors for thyroid hormone (originally identified as the cellular homolog of v-erb), 1,25 dihydroxyvitamin D_3 and retinoic acid. Striking sequence homology exists across all these receptors especially in the "C" and "E" regions.

Figure I.5 : The structure of the human oestrogen receptor.



The letters represent the functional domains (see text) and the numbers, the residues at the start and end of each domain.

3(iii) Oestrogen receptors in bone cells

Despite the well documented effects of oestrogen on bone *in vivo*, detecting oestrogen receptors in bone has proved difficult. Two approaches were used to detect oestrogen receptors; high affinity binding of radiolabelled oestradiol, or the use of specific monoclonal antibodies. An immunolocalization study by Colsten et al. (1989) failed to detect human oestrogen receptor in either primary or transformed human bone cells, or in fetal bone sections. Additionally no staining for progesterone receptor was detected in the osteosarcoma cell lines TE85C and HT396. The 29 kDa cytoplasmic product p29 which was thought to be closely related to the oestrogen receptor, was observed in high amounts in all the osteoblast-like cells studied. Chen et al (1977) determined the oestradiol receptor content in extracts of rat bone by binding ^{3}H oestradiol. Although glucocorticoid receptors were detected, no specific oestrogen binding was observed. The results from these initial studies suggested that the numbers of oestrogen receptors in osteoblast-like cells were very low.

Using high specific activity ^{125}I labelled oestradiol, oestrogen receptors were detected in rat (ROS 17/2.8) and human (HOS TE85) osteoblast-like cells (Komm et al.1988). The receptors were present in the osteoblast-like cells at a density of 200 per cell. This level of receptor is very low since several cell types including uterus cells contain several thousand high affinity oestrogen receptors per cell. The binding affinity was shown to be comparable with receptors in other tissues. Dissociation constants of 0.5nM and 1.1nM were estimated for ROS 17/2.8 and HOS TE85 cells respectively. Eriksen et al (1988) subsequently presented evidence that oestrogen receptors were present in normal human osteoblast-like cells. Bone cells were obtained from bone fragments from seven different donors and all the bone cell populations were shown to contain oestrogen receptors at an average density of 1600 per cell. Pretreatment of the cells with 10nM oestradiol increased the specific nuclear binding of progesterone *in vitro* in the majority of the strains.

There has been some data to suggest that osteoclasts might also contain oestrogen receptors. Osteoclasts were isolated from the membranous bone of 4 children, without metabolic bone disease, who were undergoing a craniotomy for either a tumour removal or trauma. Nuclear oestrogen and progesterone receptors were demonstrated by both immunolocalization and radioimmunoassay to exist in osteoclasts in this tissue. Oestrogen receptor mRNA has also been detected by Northern blot analysis in a highly purified population of avian osteoclasts (Oursler et al.1990).

In summary these findings highlight a potential mechanism whereby direct modulation of bone resorption by the sex steroids, oestrogen and progesterone, may be mediated by both osteoblasts and osteoclasts.

3(iii) Glucocorticoids

Effects of glucocorticoids on osteoblasts

In vivo administration of glucocorticoids has been shown to lower bone formation, inhibit fracture healing and cause osteoporosis in several species (see Gennam, 1985 for review). It is controversial whether the decrease in bone mass is due to a decrease in osteoblast number or activity, or a combination of the two. It is also possible that glucocorticoids have a direct inhibitory effect on osteoblast precursors. *In vitro* studies have been undertaken to address some of these questions.

Experiments with organ cultured bones give diverse and complex effects of glucocorticoid administration. The results seem to be dependent on the species and upon the time the tissue is in culture. In "bone forming" systems glucocorticoid administration gives rise to a short term increase in Type I collagen and alkaline phosphatase synthesis, followed in the long term by a decrease in cell proliferation (Canalis, 1983). This data is consistent with the idea that glucocorticoids promote an early differentiation of osteoblasts but in the long term predominantly inhibit proliferation. However this data is to some extent in disagreement with studies of

Hahn et al. (1984), who treated intact neonatal calvariae with 100nM cortisol for 24 hours. A concomitant increase in alkaline phosphatase activity and proliferation (as judged by thymidine incorporation) was observed (Hahn et al.1984).

Canalis put forward the idea that since organ cultures contain different populations of cells, the net effect of any treatment is the sum of the effects on all the different cells. Therefore he examined the effect of glucocorticoids on periosteal tissue and central non periosteal bone (osteoblast enriched) (Canalis, 1984). The data obtained suggested that mature osteoblasts are not inhibited directly, but that the predominant effect of cortisol was to diminish periosteal cell replication. It is generally thought that glucocorticoids at physiological concentrations promote the acquisition and preservation of the differentiated state of osteoblasts. Additionally, at higher concentrations or over longer term administration, glucocorticoids reduce the replication of osteoblast precursors.

Isolated osteoblasts and osteosarcoma cells contain specific high affinity glucocorticoid receptors (Chen et al. 1977; Haussler et al.1980). Treatment of osteoblast-like cells in culture with glucocorticoids decreases collagen synthesis (Peck et al.1967) and promotes RNA turnover (Peck et al.1969). Glucocorticoids can affect both PTH and 1,25 D_3 mediated events. For example, the treatment of osteoblast-like cells with glucocorticoids greatly magnifies their cAMP response to PTH (Chen and Feldman, 1978).

3 (iii) Effects of glucocorticoids on osteoclasts

Although long term *in vivo* glucocorticoid administration results invariably in bone loss, the treatment of organ cultures of bone *in vitro* with glucocorticoids gives rise to a decrease in bone resorption (Raisz et al.1972b). The mechanism of this phenomenon is not clear but may be due to a direct inhibitory effect on osteoclasts, or an inhibition of osteoclast formation. There is some evidence for both mechanisms since Suda et al (1983) showed that glucocorticoid administration to feline bone

marrow resulted in a decrease in both the generation of osteoclasts and in the activity of existing osteoclasts.

3(iv) Retinoids

Retinoids are a class of vitamin A metabolites. Some of these compounds particularly retinoic acid are thought to act as osteoblast differentiation factors. The fact that vitamin A has a role in bone metabolism has long been suspected, since is has been observed that hypervitaminosis A causes bone destruction. Vitamin A is known to stimulate bone resorption *in vivo* (Fell and Mellanby, 1952) and *in vitro* (Raisz, 1965), by an unknown mechanism which requires *de novo* RNA and protein synthesis. It is interesting to note that some of the osteoblastic effects of retinoids resemble those of 1,25 D_3, which is a structurally related steroid with a similar DNA binding receptor (Evans, 1988). Retinoic acid also enhances the binding of 1,25 D_3 to its receptor on osteosarcoma cells (Petkovich et al.1984). Retinoids have been shown to induce differentiation in some transformed cell lines including osteoblasts (Heath et al.1989).

3(v) Parathyroid hormone (PTH)

Parathyroid hormone is an important osteotropic factor with a number of target organs including bone. Within bone, PTH affects the activity of both osteoblasts and osteoclasts. PTH is a monomeric 84 amino acid polypeptide, with the receptor binding portion of the molecule consisting of the first 26 amino acid residues. It has been suggested recently that the N-terminal fragment may also confer some biological activity (Murray et al.1989). PTH is synthesized exclusively by the parathyroid gland, where its secretion is modulated by serum calcium ion concentrations. The regulation of PTH secretion has been studied *in vivo* and it has been shown that a perturbation of calcium concentration of as little as 40uM gives rise to a change in secretion rate. Since PTH is such a potent hormone, it is vital that its secretion is

closely regulated. PTH is also cleared very efficiently from the circulation by both the liver and kidney. It has been estimated that the half life for a PTH molecule is only 4 minutes. It is interesting to note that only 5 - 30% of circulating immunoreactive hormone is intact PTH, the remainder consisting of C-terminal fragments. This might reflect the efficient clearance mechanism for this hormone or that C-terminal fragments are bioactive and play a role in the activity of this hormone.

3(v) The parathyroid hormone receptor

Recently Juppner et al. (1991) used an expression cloning strategy to clone the PTH receptor. An opossum kidney cell cDNA library was prepared and transfected into a COS expression system. The transfected COS cells were then screened using radiolabelled PTH which detected positive clones. Two clones were obtained termed OK-O and OK-H, of which OK-O was shown to be a full length cDNA clone. Analysis of the clone revealed that the expressed receptor bound both PTH and PTHrP (Parathyroid related protein) with equal affinity. The binding of these ligands elicited the classical response of an increase in adenylate cyclase activity. The cloning of the calcitonin receptor was reported concurrently (Lin et al. 1991). A sequence comparison between the two receptors revealed a close homology but no similarity with other receptor families. This suggests that the receptors for these two calcium regulating hormones are related and may represent a new family of receptors.

PTH is thought to elicit its biological response through a cascade of intracellular secondary messengers. As mentioned earlier, the binding of PTH to its receptor gives rise to an increase in adenylate cyclase activity. Studies on isolated osteoblast-like cells show that the earliest detectable changes are an enhancement of adenylate cyclase activity, and an increase in cellular calcium concentration (Dziak and Stern, 1975). It is interesting to note that a prolonged stimulation of PTH on osteoblast-like cells results in a desensitization to PTH and a diminished cAMP response. The PTH receptor has been detected on osteoblasts, lining cells and osteocytes *in situ* by ^{3}H-

PTH autoradiography (Barling and Bibby, 1985). Osteoblast-like cells in culture have been also shown to express PTH receptors (Pliam et al.1979).

3(v) The effect of PTH on osteoblasts

PTH has been reported to exert a variety of effects on osteoblasts which are dependant on the dose and upon the length of administration. At doses of greater than $1x10^{-9}M$, PTH stimulates osteoblast-like cell proliferation *in vitro* (MacDonald et al.1986a). However, some osteosarcoma cell lines differ in this property and their replication is inhibited with doses of greater than $1x10^{-10}M$ (Partridge et al.1985). This may be due to the fact that isolated osteoblast-like cells and osteosarcoma cells are at different differentiation stages. PTH treatment also modulates the synthesis of osteoblastic proteins in a dose dependant manner. For example, the treatment of osteoblast-like cells from chick calvariae with a low dose of PTH results in an increase in alkaline phosphatase activity, whilst high doses suppress activity (Hall and Dickson, 1985). *In vivo* PTH administration gives rise to a decrease in collagen synthesis in short term organ cultures (Dietrich et al.1976). However long term administration of PTH at intermittent or low doses stimulates bone formation (Guines-Hey and Hock, 1984).
It is interesting to speculate why PTH shows such varying effects at different doses and lengths of administration. The experiments undertaken might represent different physiological levels of this hormone in the body. Low levels of circulating PTH may result at the end of a resorption period and might then result in a proliferation of osteoblasts to ensure efficient bone formation. The results of experiments where PTH is administered intermittently should be considered as important findings, as this probably mirrors more closely the pulses of secretion of this hormone in the body.

Table I.2: The effect of PTH and PTHrP on bone resorption

Agent	Dose	Culture system	Effect	Reference
PTH	0.1 - 25 nM	Fetal rat long bones in organ culture.	⬆ in resorption	Raisz et al.1990
PTHrP	0.1 - 25 nM	Fetal rat long bones in organ culture.	⬆ in resorption	Raisz et al.1990
PTH	0.2 - 20 nM	Neonatal rat osteoclasts on bovine cortical bone slices.	⬆ in resorption due to an ⬆ in number of resorption pits	Murrills et al.1990
PTHrP	0.2 - 20 nM	Neonatal rat osteoclasts on bovine cortical bone slices.	⬆ in resorption due to an ⬆ in number of resorption pits	Murrills et al.1990
PTH	1 pM	Embryonic chick tibia in organ culture	⬆ in resorption	Howard et al.1981
PTH	40 nM	Osteoclast-like cells from neonatal rat long bone on cortical bone slices	No effect on bone resorption.	McSheehy and Chambers, 1986
PTH	40 nM	Same as above plus osteoblast like cells	4 fold increase in resorption.	McSheehy and Chambers, 1986
Supernatants from PTH treated obs.	n/a	Rat macrophages on devitalized particles of rat bone.	⬆ bone resorption.	Perry et al.1989
Supernatants from PTH treated obs.	n/a	Fetal bone rudiments of rat radii in organ culture.	⬆ bone resorption.	Perry et al.1989
Supernatants from PTH treated obs.	n/a	Isolated chick osteoclasts on devitalized particles of rat bone.	⬆ bone resorption.	Perry et al.1989

obs. = osteoblast. n/a = not applicable ⬆ = increase.

3(v) The effect of PTH on osteoclasts

PTH has also been shown to affect osteoclast activity. Table I.2 summarises its effects on bone resorption *in vivo* and *in vitro.* PTH is a potent resorptive agent which gives rise to two phases of osteoclast responsiveness. They appear to involve a short term activation of mature osteoclasts and immediate precursors, followed by the long term recruitment of newly formed osteoclasts. PTH administration *in vivo* results in an increase in the number of osteoclasts per volume of bone tissue. This effect can be seen as early as 2 hours after treatment in mice, and 8 hours in rats (Tatevossian, 1973; Baron and Vignery, 1981a). It has been shown that DNA synthesis is not required for this short term effect. Therefore it is thought that the increase in osteoclast numbers results from the fusion of immediate osteoclast precursors. Longer term administration of greater than 3 days also gives rise to an increase in osteoclast numbers, presumably by stimulating the differentiation of osteoclast progenitor cells. PTH has also been shown to stimulate the resorptive activity of existing osteoclasts. It increases motility, ruffled border areas, TRAP, carbonic anhydrase and lysosomal enzyme activity (Holtrop et al.1974; Chambers et al.1984; Hammarstrom et al.1971).

Isolated osteoclasts have been shown to be unresponsive to PTH, and no PTH receptors have been detected on osteoclasts (Silve et al.1982). It is thought that PTH exerts its effects on osteoclasts via osteoclast precursors/progenitors, or other cells in the bone microenvironment such as osteoblasts.

3(vi) Calcitonin

Calcitonin is a small (32 amino acid) peptide almost exclusively produced by the thyroid gland, although some immune cells and neurones are capable of its synthesis. There is a tissue specific transcription of the calcitonin gene to give two different

mRNA species encoding for calcitonin and calcitonin gene related peptide (CGrP) (Deftos and Roos, 1989).

The major skeletal action of calcitonin is the inhibition of osteoclast mediated bone resorption. *In vivo* administration of calcitonin results in the detachment of osteoclasts from the bone surface and a decrease in the number of osteoclasts present. This occurs in rat rib bone within 30 minutes (Baron and Vignery, 1981). In organ culture systems calcitonin inhibits not only endogenous resorption, but that stimulated by PTH, prostaglandin E (PGE) and 1,25 D_3 (Raisz, 1976).

In vitro bone resorption models have illustrated an important feature of calcitonin action. There is a reappearance of resorption after the initial suppression within 24 - 48 hours even though calcitonin is still present. This phenomenon is termed "escape" (Wener et al.1972). "Escape" is thought to be due to a down regulation step at the cellular level. There are several mechanisms by which "escape" could conceivably occur. They include a reduction in the affinity /calcitonin receptor level, or a change in cell dynamics with the emergence of calcitonin insensitive osteoclasts. Isolated osteoclasts in culture respond to calcitonin within minutes of treatment by undergoing major shape changes. The response is typically characterised by a reduction in ruffled border area, retraction of cytoplasmic extensions and a decrease in motility (Chambers et al.1984).

Cyclic AMP has been proposed as a secondary messenger for calcitonin action in osteoclasts. Calcitonin has also been shown to decrease cellular calcium ion concentrations in a population of osteoclast-like cells *in vitro* (Schlossman et al.1982). Calcitonin also has some anabolic effects in bone. Normal human osteoblasts proliferate, and increase alkaline phosphatase and type I collagen synthesis in response to calcitonin treatment (Farley et al.1989).

3(vii) The role of cytokines in the control of bone remodelling

The role of cytokines in bone remodelling has been the subject of much research in the last 20 years. Many different experimental approaches have been used to try to identify the components of a cytokine bone network, and define their different activities. Studies using culture models of bone cells to monitor the effects of isolated cytokines have been very prevalent. Unfortunately ,although a large amount of data has been published from these studies, the literature is very confused and controversial. This confusion is due to the fact that different investigators have used bone cells obtained from different animal species and culture protocols. The basic nature of the bone cells vary, some bone cells are from primary cultures whereas others are transformed cell lines derived from osteosarcomas. The source of cytokine is also a cause of discrepancy. The degree of purity varies from cell supernatants to purified proteins, to recombinant cytokine. In addition the doses of cytokine and duration of treatment are not standard between different studies. For this reason the following account reviews the data obtained from *in vivo* studies, organ cultures and cells directly obtained from the organ cultures discussed. Although these studies do not pinpoint the exact cellular interactions that take place, they highlight potential roles of the cytokines in bone remodelling *in vivo*. The following section focuses on the roles of two important osteotropic cytokines TGFβ and IL-1, since these two cytokines are the main subject of this thesis.

3(viii) The effects of IL-1 and TGFβ on bone resorption

The initial finding that prompted the interest in cytokines as a bone resorbing agents came from studies on the mechanism of peridontal disease. Antigen extracts from dental plaque were used to stimulate leukocytes from patients with peridontal disease. It was observed that the degree of *in vitro* stimulation was directly proportional to the severity of disease. Since severe peridontal disease was associated with increased

leukocyte infiltration and bone loss, the resorption activity of the supernatants was assayed using ^{45}Ca radiolabelled fetal rat bones. The supernatants were shown to contain a soluble factor which induced bone resorption in a dose dependant manner (Horton et al.1972). A microscopic analysis of the bones after treatment with this soluble factor showed that the bone contained increased numbers of osteoclasts as well as large areas of resorption. The soluble factor was tentatively named osteoclast activating factor (OAF).

These observations prompted the search for the identity of OAF. Gowen et al. (1983) demonstrated that supernatants obtained from the monocytes of a patient with chronic myelomonocytic leukaemia stimulated bone resorption of neonatal mouse calvarial bones. The supernatants were pooled and chromatographed on a molecular sieve column followed by isoelectric focussing to reveal an active component with a molecular weight of 12 - 18 kDa. The bone resorbing agent was shown to be active in the leukocyte activation (LAF) assay which detects interleukin 1 activity. Therefore OAF was proposed to contain as one of its components an "interleukin-1-like factor". The identity of this "interleukin-1-like factor" was later confirmed to be IL-1 by Dewhirst et al. (1985) who purified the factor by gel filtration and HPLC to obtain a single protein band on PAGE. The protein was shown to have the same amino acid terminal sequence, isoelectric point and molecular weight as IL-1. However it is important to note that the original OAF may have contained other bone resorbing agents since the purification protocol was designed to purify an "interleukin-1-like activity".

Shortly after this finding, it was demonstrated that purified pig IL-1 stimulated bone resorption in the mouse calvariae bone resorption assay (Heath et al.1985). The resorption was partially inhibited by indomethacin which suggested that it may be mediated by local prostaglandin production. Further studies using recombinant murine IL-1 confirmed its potent action as a bone resorbing agent (Gowen and Mundy, 1986). Resorption occurred after a 48 hour lag phase with very low doses of

recombinant IL-1 (as little as 23 pg/ml). The resorption occurred after a stimulation of as little as 2 hours, and was shown to be insensitive to indomethacin treatment but inhibited by interferon gamma. Similar to the original observations of Horton, an increase in the numbers of osteoclasts present and an enlarged marrow cavity was observed. The results suggested that IL-1 induced some early event which lead to the increase in osteoclast formation from progenitor or precursor cells.

The previously described organ culture experiments prompted an extensive *in vivo* study of the action of IL-1 in normal mice (Boyce et al.1989). The mice were injected subcutaneously with human recombinant IL-1 once daily for 3 days over the right calvaria. The calvariae were then examined for the effects of IL-1 administration on morphology during the subsequent 4 week period using quantitative histomorphometry. The IL-1 treatment was associated with a transient elevated plasma level of calcium 24 hours after the last injection . After 3-4 days of treatment, most of the bone on the injected side was resorbed. These initial events were insensitive to indomethacin treatment, in contrast to those over the longer term where most of the resorbed bone was replaced by increased amounts of new bone.

It was demonstrated that soluble IL-1 was not the only form which was capable of inducing bone formation. A murine macrophage cell line was stimulated with LPS and fixed to provide a membrane bound form of IL-1 (Nishihara et al.1989). This membrane bound form, was effective in stimulating bone resorption in organ cultures of mouse neonatal calvariae. The effect was blocked by neutralizing antibodies, and inhibited with calcitonin and indomethacin.

Similar organ culture studies were undertaken with transforming growth factor beta (TGFβ). Tashjian et al (1985) demonstrated that TGFβ stimulated bone resorption in cultured mouse calvariae. Significant resorption, mediated via prostaglandin production occurred with as little as 0.2 ng /ml of TGFβ. Since epidermal growth factor (EGF) had been shown to act synergistically with TGFβ during the transformation of normal kidney fibroblasts (Roberts et al.1981), it was used in

conjunction with TGFβ in the resorption assay but no synergism was observed. Pfeilschifter et al. (1987) investigated the bone resorbing activity of TGFβ in the alternative rat long bone organ culture model. In contrast to previous findings TGFβ was shown to inhibit bone resorption. Since the effect was similar to that of hydroxyurea (a DNA synthesis inhibitor), it was assumed that TGFβ acted by preventing the formation of osteoclasts. TGFβ treatment was also shown to inhibit bone resorption during prolonged culture with submaximal doses of IL-1, PTH and 1,25 D_3. These data were supported by the fact that TGFβ inhibits the formation of osteoclasts from precursors in cultures of bone marrow *in vitro* (Chenu and Pfeilschifter, 1988).

In a study similar to that of Boyce et al. (1989), TGFβ was injected subcutaneously over the calvariae of normal mice. Increased numbers of large multinucleated osteoclasts were detected in the marrow spaces (Marcelli et al.1990). Bone resorption was enhanced over a 3 - 5 day period. However unlike IL-1 stimulated resorption, no degradation of the periosteal layer was seen. Indomethacin inhibited the increased appearance of osteoclasts in the marrow space, suggesting that the response was prostaglandin mediated.

Since cytokine mediated bone resorption had been suggested to be both prostaglandin dependent and independent, Garrett and Mundy (1989) investigated the effect of different assay protocols on resorption. The data obtained showed that events during the preculture period were important in modulating the later effects of any factor on bone resorption. It was shown that osteoclast numbers increased after 24 hours in control media due to increased endogenous production of prostaglandins. Additionally the numbers of osteoclasts fell when the calvaria were transferred to fresh control media for 72 hours. When IL-1 was added after the preincubation period, osteoclast numbers were maintained and indomethacin had no effect on the increased resorption. Furthermore IL-1 did not seem to promote precursor proliferation since

hydroxyurea had no effect on the response. Therefore it was suggested that IL-1 stimulated bone resorption by acting at a later stage of precursor differentiation.

3(ix) The effects of IL-1 and TGFβ on bone formation

There are no reports of IL-1 stimulating bone formation *in vitro*, although in the study by Boyce et al. (1989) there was a transient increase in bone mass in IL-1 treated mice. After the initial resorption, bone was replaced in an accelerated manner and 9 days after treatment the bone volumes of the IL-1 stimulated calvariae were significantly higher than those of saline treated controls. It is unlikely that this finding reveals a role for IL-1 in bone formation, but rather that the coupling mechanism in the calvariae was stimulated so that bone homeostasis was achieved.

In vitro studies suggest that IL-1 may have an anabolic role in bone metabolism. IL-1 treatment for a 24 hour period has been shown to stimulate DNA synthesis in rat calvariae. A large increase in total protein synthesis, of which collagen expression was significantly increased, was also observed following IL-1 treatment (Canalis, 1986). In the same study, it was shown that high doses or longer exposure times of IL-1 inhibited the expression of collagen. It has been reported that the prolonged exposure of fetal rat osteoblasts *in vitro* to IL-1 inhibits the formation of mineralized bone nodules (Stashenko et al. 1991). This data suggests that bone is exquisitely sensitive to local IL-1 concentrations and the effect of this cytokine is dependent on both dose and time of exposure amongst other factors.

Many studies have reported the striking anabolic effect of TGFβ treatment on bone. The initial study monitored the *in vivo* stimulation of bone formation in rat calvariae injected daily for 12 days over the periosteum of the right pareital bones (Noda and Camilliere, 1989). TGFβ was shown to increase bone formation in a dose dependent manner, with the thickness of the parietal bone increasing by two fold. The bone formed after 5 days was shown to be immature woven bone. The second isoform of TGFβ ($TGF\beta_2$) was also shown to have similar effects. It is important to note that the

effect of TGFβ was not systemic, but occurred in a very localized manner at the site of injection, and was not evident in any other bones. In a similar study by Joyce et al. (1990), TGFβ was shown to be an important factor for the chondrogenesis and osteogenesis of rat long bone. The periosteal regions of femurs in new born rats, were injected daily with $TGF\beta_1$ or $TGF\beta_2$ for 14 days. The femurs were subsequently removed at periods after the cessation of treatment and assessed for any morphological changes. Three days after the cessation of treatment, the mesenchymal cells in the periosteum and endosteum were seen to proliferate and there was also an increase in fibrous tissue at the site of injection. At day 5, cartilaginous matrix was detected at the injection site, and by day 15 there was a large mass of cartilage at the site of injection. This mass of cartilage was flanked by intramembranous bone formed in the cortical region. Over the longer term (3 - 4 weeks later) the cartilage was replaced with bone by endochondral ossification. $TGF\beta_2$ was shown to be more active than $TGF\beta_1$ in stimulating bone mass *in vivo* , with greater than 300% more bone formed at comparable doses. With both isoforms the type of tissue formed was dependent on the dose of TGFβ. At low doses (20ng/day) proportionately more bone was formed, whilst at high doses (200ng/ml) more cartilage was evident. This finding was particularly interesting in light of the fact that $TGF\beta_1$ is isolated in a 4:1 ratio to $TGF\beta_2$ from mature bone. This data suggests that TGFβ is an important regulatory factor in the mechanism of proliferation and differentiation of mesenchymal bone precursor cells.

A study by Beck et al. (1991) highlighted the importance of the perichondrium in TGFβ stimulated bone formation. Recombinant human $TGF\beta_1$ ($rhTGF\beta_1$) was shown to accelerate the onset and increase bone formation in a soft tissue wound healing model. A single dose of 25-100ng $rhTGF\beta_1$ was applied to the adjacent cartilage in a full thickness skin wound. Bone formation was detected at the site of application after 21 days and peaked after 42 days. There were two phases of tissue repair, initially $TGF\beta_1$ induced the differentiation of perichondrial cells to osteoblasts

which synthesized osteoid onto a cartilage template. Secondly, continued bone formation occurred until remodelling was initiated, during which the cartilage and newly formed bone was eroded to form a marrow cavity. No bone formation occurred when the perichondrium was intact. Only when it was removed and rhTGFβ1 made direct contact with the damaged perichondrium and cartilage, was bone formation induced at the wound margin.

In summary, the consensus of the data suggest that IL-1 is a very important factor in promoting bone resorption and has variable effects on bone formation. The bidirectional effect of TGFβ (which is also seen in other systems) may be dependent on the prevailing conditions in the microenvironment of the exposed cell, the dose and duration of TGFβ and the presence of other growth factors.

Other cytokines that modulate bone formation or resorption

Table I.3 outlines the effects of other cytokines that modulate bone resorption or formation. For the purpose of conciseness, the cytokines have been divided into 4 groups; inflammatory cytokines, growth factors, colony stimulating factors and resorption inhibitors .

3(x) "Inflammatory" cytokines

"Inflammatory" cytokines are defined for this review as those factors which give rise to fever, and induce the synthesis of acute phase proteins. They include tumour necrosis factor alpha (TNFα), interleukin 6 (IL-6) and the IL-1s which have been discussed previously.

TNFα (so named as it is a cytotoxic agent for a number of tumour cell lines) is a monomeric 17 kDa protein derived primarily from monocytes. As with many other cytokines, TNFα has many diverse biological effects including stimulating T cell growth and differentiation (Beutler and Cerami, 1989) and the induction of adhesion molecules (Furie and McHugh, 1989). The most striking feature of the osteotropic

effects of TNFα is that it shares so many activities with IL-1. TNFα like IL-1, induces bone resorption (Bertolini et al.1986) presumably by promoting osteoclast formation (Pfeilschifter et al.1989) and stimulating prostaglandin production. In addition, it promotes osteoblast proliferation (Gowen et al.1988). TNFα also stimulates osteoblasts to release IL-1 and IL-6 (Hughes et al.1988; Littlewood et al. 1991), and therefore acts to promote a cytokine network in bone.

IL-6 also has many diverse actions; it promotes the maturation of B cells to immunoglobulin producing plasma cells (Kishimoto, 1985; Kishimoto and Hirano, 1988) and acts as a growth factor for many hybridomas and myeloma cell lines (Van Damme et al.1987). IL-6 is a glycoprotein that is synthesized in a pro-form and cleaved to form an active 26 kDa protein. A variety of cells synthesize IL-6 including stimulated monocytes, endothelial cells and fibroblasts (Aarden et al.1985). The role of IL-6 in bone metabolism has not been fully elucidated as it does not seem to stimulate the production of any cytokines or resorptive agents by human osteoblast-like cells (Littlewood et al.1991a). The role of IL-6 in bone resorption is controversial, and it has been reported to have no effect (Al-Humidan et al.1991), or stimulate (Lowik et al.1989) bone resorption, depending on the organ culture system used. IL-6 is an important regulator of hematopoiesis (Wong et al.1988), and is synthesized by osteoblast-like cells in culture (Littlewood et al.1991b). It has been postulated to have a role in promoting osteoclast formation (Kurihara et al.1990). It is thought that IL-6 may act in conjunction with other stem cell growth factors to promote the differentiation of hematopoietic stem cells to give an increased pool of osteoclast progenitor cells. The evidence for this scenario in bone is very circumstantial, but includes the observation that IL-6 has a colony stimulating factor (CSF)-like activity and stimulates granulocyte/ macrophage colony formation from bone marrow cells (Kurihara et al.1990).

3(xi) Growth factors

Growth factors are defined as extracellular proteins that affect cell proliferation and/ or differentiation. They are often classified according to their origin (eg. PDGF- platelet derived growth factor) or their effect (eg. TGFα- transforming growth factor alpha). The following growth factors are all reported to have osteotropic actions, but it must be borne in mind that these effects might be secondary to their primary roles in growth or repair.

Epidermal growth factor (EGF) is a 6 kDa polypeptide originally isolated from the mouse submaxillary gland and human urine (Cohen, 1983). Receptors for EGF have been identified on osteoblast-like cells including those from mouse calvariae and osteosarcoma cell lines (Shupnick et al.1980). EGF has been reported to have opposing effects on bone since it increases bone resorption, but also increases proliferation of osteoblasts (Raisz et al.1980; Tashjian and Levine, 1978; Canalis and Raisz, 1979). EGF is thought to promote an undifferentiated osteoblast phenotype since it reduces alkaline phosphatase and type I collagen synthesis in osteoblasts from different species (Shupnik and Tashjian, 1981).

EGF shares the same receptor as transforming growth factor alpha (TGFα). TGFα is a 50 amino acid polypeptide found in tumour cell culture supernatants. (Todaro et al.1980). It is thought to be a possible mediator of hypercalcaemia associated with neoplasms (eg humoral hypercalcaemia of malignancy). As expected, TGFα mimics the effects of EGF on bone. Signal transduction for both growth factors is thought to occur by the phosphorylation of cellular proteins, including the receptor, by specific protein kinases.

Fibroblast growth factor (FGF) is isolated from brain tissue and pituitary gland (Bohen et al.1985). It is a potent mitogenic cytokine and promotes the proliferation of many cell types including mesenchymal, endothelial and osteoblast-like cells (Canalis and Raisz, 1980). PDGF is another mitogenic protein with opposing effects

on bone. It enhances bone resorption ostensibly via local prostaglandin release (Canalis, 1981) and stimulates osteoblast proliferation (Tashjian et al.1982). PDGF is not a single factor but rather a family of 30kDa heterodimers consisting of combinations of both α and β subunits. In contrast to EGF and FGF, PDGF increases the synthesis of type I collagen which might also reflect its role in wound healing (Canalis et al.1989).

There has been a large amount of interest in the role of insulin like growth factors (IGFs) in bone. Two forms have been isolated from bone matrix, namely IGFI and IGFII. Their effects in bone are thought to be largely anabolic since both have been shown to induce osteoblast mitogenesis in culture (Schmid et al.1983), and stimulate the synthesis of matrix proteins including type I collagen (Canalis, 1980). Their biological activities are modulated by a series of specific high affinity binding proteins which are also synthesized by osteoblasts (Mohan and Baylink, 1991). The synthesis of these binding proteins is linked to the expression of other growth factors. It has been shown that FGF, TGFα, PDGF, and EGF are potent inducers of the synthesis of certain IGF binding proteins by osteoblast-like cells (Chen et al.1991).

3(xii) Colony stimulating factors

Colony stimulating factors are members of the hematopoietic growth factor group. There are at least 5 members in this group, however only granulocyte-macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF) will be discussed in this review, since they have been shown to target bone cell precursors.

GM-CSF is produced by a wide range of cell types including activated T lymphoblasts, monocytes, stimulated fibroblasts and endothelial cells (Seelentag et al.1987). M-CSF is also secreted by monocytes, but is mainly produced by anchorage dependent cells including fibroblasts, keratinocytes, and endothelial cells.

Osteoblasts from several species have been shown to produce both factors *in vitro* when stimulated with LPS or PTH (Horowitz et al.1989).

The data concerning the effects of GM-CSF on bone resorption is conflicting. GM-CSF has been shown to increase the formation of osteoclasts from bone marrow cell cultures (MacDonald et al.1986b). In contrast it has been reported to reduce the the rate of appearance of mature osteoclasts in the fetal mouse long bone resorption model (Lorenzo et al.1987). In addition, studies with mouse bone marrow adjacent to slices of cortical bone, reveal that the addition of GM-CSF not only inhibits basal bone resorption, but also 1,25 D_3 stimulated resorption (Hattersley et al.1988). M-CSF has also been reported to enhance the formation of osteoclasts in long term marrow cultures. Further enhancement could be achieved by the addition of 1,25 D_3 (MacDonald et al.1986b). M-CSF is also active in bone resorption in certain cases, resorption is stimulated in cultures of embryonic metacarpal bones (Horowitz and Jilka, 1991) but not 6 day old calvariae. This suggests that M-CSF stimulates osteoclastogenesis but does not affect mature osteoclasts.

Studies on the osteopetrotic (op/op) mouse yield the most convincing data that M-CSF is involved in osteoclast development. These mice are congenitally osteopetrotic due to a homozygous recessive mutation which gives rise to a deficiency in osteoclasts and macrophages (Wiktor-Jedrzeczak et al.1982). The osteopetrotic symptoms are not alleviated by the transfer of normal bone marrow which suggests that the defect was not the result of a lack of precursor cells, but rather an incorrect hematopoietic microenvironment. Further investigation showed that the phenotype was due to a lack of functional M-CSF. Normal osteoclast and macrophage function including bone resorption could be restored by administering large doses of M-CSF. Analysis of the M-CSF gene in these mice revealed that a thymidine base had been inserted into the gene 3 prime to the initiation codon, which gave rise to a TGA stop codon further downstream of the gene (Yoshida, 1990). Northern blotting showed that instead of the two mRNA species that were normally seen, cells from the op/op mice only

contained a single small transcript representing a secreted form of the protein. Since the M-CSF was not functional it is assumed that either the secreted form was defective or that a membrane bound form of M-CSF was necessary for activity.

There is little evidence that osteoblasts respond to CSFs but Dedhar et al. (1988) reported that the human osteosarcoma cell line MG-63 proliferates in response to GM-CSF. In the same study the more mature variant cell line MG-63.3a failed to proliferate. This raises the possibility that CSFs may be active differentiation agents for immature osteoblasts.

3(xiii) Resorption inhibitors

Interferons are a class of cytokines produced by lymphocytes and macrophages following stimulation, and are thought to be important in the antiviral response. Of the known interferons interferon gamma (IFN-γ) acts to promote the immune and inflammatory responses of lymphocytes and macrophages. IFN-γ has been shown to selectively and completely inhibit only cytokine (IL-1, TNFα, TNFβ) stimulated bone resorption (Gowen et al.1986), but not 1,25 D_3 or PTH stimulated resorption under the same conditions. Three different mechanisms have been proposed for IFN-γ inhibition of bone resorption. Firstly prostaglandin mediated bone resorption may be inhibited by IFN-γ acting to block prostaglandin synthesis. There is some evidence to suggest that this occurs in neonatal calvariae (Hoffman et al.1987). Secondly IFNγ may have a direct calcitonin like effect, or thirdly it might inhibit osteoclast recruitment or formation.

IFN-γ also has effects on bone formation, as it has been shown to inhibit type I collagen synthesis in fetal rat calvariae (Smith et al.1987) and has an additive effect with TNFα and TNFβ when bone is exposed to both.

Recently some data has been presented which suggests that the T cell product, interleukin 4 (IL-4) may have a role in bone metabolism. IL-4 has the ability to induce monocyte-macrophage differentiation from precursor cells (Galanaud et al.1990).

Since IL-4 induces differentiation of precursors, from a simplistic view it could be assumed that it might promote osteoclast formation. However its role in bone resorption seems to be inhibitory. Fetal rat long bones exhibited no increases in bone resorption after 3 days when treated with recombinant murine IL-4 (rmIL-4). However significant inhibition of basal resorption occurred after 5 days (Watanabe et al.1991). In addition IL-4 inhibited both IL-1, prostaglandin and PTH stimulated bone resorption in a dose dependent manner. Due to the relatively long period before IL-4 exerts its inhibitory actions on resorption, it seems likely that it acts to decrease osteoclast formation. This might not be a direct effect, as it is possible that IL-4 acts by decreasing the precursor pool available for osteoclast formation by promoting differentiation along the macrophage monocyte lineage. In another study by Watanabe et al (1990) rmIL-4 was shown to be effective in abrogating PTHrP induced hypercalcaemia. A histological examination of the bone revealed a decrease in endosteal osteoclast formation.

Table I.3: The effect of cytokines on bone metabolism

	Osteoclast formation	Bone resorption	Osteoblast growth	Bone formation	References
Inflammatory cytokines					
IL-1	↑	↑	↑		Gowen, 1991
IL-6	↑	↑↓	→	→	Lowik et al.1989; Al-Humidan et al.1991; Littlewood et al.1991
TNFs	↑	↑	↑		Gowen, 1991
CSFs					
M-CSF	↑	↑↓			Horowitz and Jilka, 1991
GM-CSF	↑↓	↓	↑		Horowitz and Jilka, 1991
Growth factors					
TGFβ		↑↓	↑	↑	Dieudonne et al.1991;Hattersley and Chambers 1991; Marcelli et al.1990.
IGFs			↑	↑	Schmid and Ernst, 1991
PDGF		↑	↑↓		Rodan and Rodan, 1991
TGFα/EGF	↑	↑	↑		D' Souza and Ibbotson, 1991
Resorption inhibitors					
IL-4	↓	↓	↑		Watanabe et al.1991;Watanabe et al.1990; Lacey et al.1991
IFN-γ	↓	↓	↓	↑↓	MacDonald, 1991

↑ = increase

↓ = decrease

→ = no effect

3(xiv) Summary

The previous section has reviewed the current data available for the actions of different groups of cytokines in bone. Much of the data are obtained from experiments where different cytokines are used in isolation. Whilst the data obtained may highlight some functions of the cytokine, it is unlikely that the effects will mirror the *in vivo* state where whole networks of cytokines interact. Hopefully with the advent of more molecular biology techniques, where several parameters can be studied at once, a greater understanding of the complex actions of cytokines in bone could be achieved.

In summary, this introduction has outlined the structure, and major components of bone. The mechanisms by which bone is thought to be formed and remodelled have been described. In addition, the available data concerning the role of systemic hormones and growth factors has been reviewed. Our understanding of bone metabolism, particularly with reference to human bone is at a very preliminary stage. Only by characterising the complex events that control bone remodelling will it be possible to address the problems of metabolic bone disease. The following chapters report the findings of several indepth studies of the role of two cytokines IL-1 and TGFβ in human bone metabolism. It is hoped that the studies will contribute to the understanding of the complex cytokine network present in bone.

CHAPTER II : MATERIALS AND METHODS

II.1 MATERIALS

All chemicals were of the purest grade available and were obtained mainly from Sigma.

The sources of specialist materials are listed below.

Antibodies and cytokines

Recombinant TGFβ - British Biotechnology

Recombinant IL-1β - Glaxo (Greenford)

Recombinant TNFα - Glaxo (Greenford)

Anti TGFβ polyclonal antibody - British Biotechnology

Anti TGFβ monoclonal antibody - Celtrix (USA)

Anti IL-1β monoclonal antibody - Roussell (Switzerland)

Culture materials

Gibco - Modified Eagles medium (MEM), fetal calf serum, trypsin/EDTA solution.

Oxoid Ltd - phosphate buffered saline

Sigma - sodium selenite, insulin

Sterilin - sterile tissue culture grade plastics, cell scrapers

Miscellaneous biochemical reagents

Biorad - electrophoresis grade acrylamide

Ilford - chemicals for film development

Kodak - film

British biotechnology - TGFβ fluorokine receptor kit, IL-1β fluorokine receptor kit

Molecular probes, Triangle Park N.C. - FITC conjugated beads for FACS analysis.

Molecular reagents

Restriction enzymes and Taq polymerase were from NBL. Modifying enzymes (Klenow, SP6 polymerase, RNase A, T7 polymerase) were from Mannheim-Boehringer.

Amersham - LM-1 emulsion, Hybond N+

Oxoid Ltd - yeast extract, bactotryptone, bactoagar

Sigma - MOPS, formamide, salmon sperm DNA (RNAse free)

Stratech Scientific ltd - Geneclean kit

GIBCO - Phenol, formamide (RNA grade)

Plasmids - pgemil1beta, pGHTGFβ-27 and pGEM-IRAP-P5 were kind gifts from Dr. A Shaw, Glaxo Geneva. pOP/10 was a kind gift from Dr. M Young NIH Bethesda Maryland.

Radioisotopes and related materials

Amersham International plc : deoxycytidine - [α-^{35}S] thiotriphosphate, deoxycytidine 5'-[α-^{32}P] triphosphate, [α-methyl ^{3}H] thymidine.

II.2 CELL BIOLOGY METHODS

II.1(i) Human bone cell culture.

The human osteoblast-like cells were derived from explants of trabecular bone obtained at surgery as previously described (Beresford et al.1984; Beresford et al.1986). After four to six weeks in culture the cells were trypsinized, pooled and then seeded in 9 cm^2 petri dishes at one million per dish. For the serum-free experiments the cells were incubated in serum-free MEM + 0.1% BSA for 24 hours prior to the experiment. These cultures have been previously extensively screened for macrophages and monocytes using a panel of monoclonal antibodies and both these cell types have been shown to be absent (Skjodt et al.1989).

II.1(ii) Interleukin 1 bioassay

The level of expression of IL-1 activity was assessed using the $D_{10}N_4M$ bioassay. $D_{10}N_4M$ is a murine T cell line which proliferates in response to IL-1 in the presence of IL-2 (Hopkins and Humphreys, 1989). Samples to be assayed were dispensed at 100ul per well. After a 72 hour incubation the cells were pulsed with [α-methyl ^3H] thymidine (1uCi per well) for a further 6 hours. The extent of proliferation and hence IL-1 bioactivity present in the samples was determined by harvesting the cells, scintillation counting and comparing with a standard curve. The bioassay was sensitive down to 0.2 pg /ml IL-1.

II.1(iii) Cell proliferation assay

Human osteoblast-like cells were trypsinized and harvested from the $9cm^2$ petri dishes. Cells were then seeded at a density of $2x10^5$/ well in a 48 well Costar tissue culture plate and left overnight in a 5% CO_2 incubator at 37°C to adhere. The following day the treatments were added to the wells in triplicate, and the cells incubated for a further 24 or 48 hours depending on the agents. After this time the cells were "pulsed" for 6 hours with [α-methyl ^3H] thymidine (1uCi per well) to assess the proliferation. At the end of the pulse period the cells were washed extensively with cold PBS to remove any surface bound [α-methyl ^3H] thymidine. The DNA was then harvested by lysing the cells with 0.1% SDS/PBS on ice for 5 min and spoting the lysate onto DE18 filter paper. The filters were washed 3 times in an excess of 0.5M sodium phosphate solution to wash away unincorporated [α-methyl ^3H] thymidine. Tritiated thymidine incorporation was then estimated by liquid scintillation counting.

II.1(iv) Immunolocalization of cytokines in cells

Human osteoblast-like cells were plated onto 10 well multispot slides which had previously been washed in 70% ethanol and autoclaved. The cells were seeded at a

density of 3000/spot and settled overnight at 37°C in a 5% CO_2 atmosphere. The cells were treated with 200 pg/ml IL-1α to stimulate IL-1β expression, or 10^{-8}M 1,25 dihydroxyvitamin D_3 for TGFβ expression, for 24 and 48 hours respectively. To stop cytokine secretion cells were treated with 0.5uM monensin at the start of the stimulation peroid. After the stimulation the cells were washed briefly in PBS and fixed in 4% paraformaldehyde/PBS for 5 mins. The cells were then washed with TBS three times for 5 min to remove any traces of paraformaldehyde. The slides were immediately stained, or stored air dried for up to 1 month at -20°C. A treatment with 0.1% Triton X 100 was performed for 5 min to increase the accessibility of the antibodies. Residues of Triton X 100 were removed with TBS washes. The primary antibodies in 5% horse serum were layered onto the cells for 30 min in a humidified chamber. Excess antibody was removed with TBS washing, the cells were then incubated with a secondary antibody which was conjugated to alkaline phosphatase. After the excess secondary antibody was removed by washing in TBS, an alkaline phosphatase substrate (2mg/ml Napthol AS-MX phosphate in 2% N,N-dimethylformamide, 0.1 M Tris pH 8.2, 100 mM levamisole, 1mg/ml Fast-Red TR salt) was added to produce a coloured reaction product. 100mM levamisole was found to be the optimal concentration to inhibit endogenous alkaline phosphatase activity. The slides were mounted in 90% glycerol, 10% TBS and sealed. In some cases the cells were reacted with a different coloured substrate to show endogenous alkaline phosphatase activity, prior to antibody staining.

II.1(v) Alkaline phosphatase staining

The sections were air dried prior to alkaline phosphatase staining. The reaction buffer (0.2 mg/ ml Napthol AS-MX phosphate in 2% N,N dimethyl formamide, 0.1 M Tris pH 8.2, 1 mg/ml Fast Blue BB salt) was prepared immediatly before staining, and filtered directly onto the sections. The reaction was carried out at room temperature for 2 - 5 min until a blue precipitate was visible. The sections were then air dried, mounted in 90% glycerol, 10% PBS and sealed.

II.1(vi) Tartrate Resistant Acid Phophatase staining (TRAP)

TRAP activity was assayed by the standard napthol AS-BI phosphate post-coupling method, using Fast Garnet GBC as the coupler. The reaction was carried out at 37°C in a water bath. The sections were incubated with prewarmed reaction buffer (0.25M sodium citrate pH 4.5, 0.5 mg/ml Napthol AS-BI phosphate) for 5 -10 min. The napthol reaction product was then reacted with Fast Garnet to give a red coloured stain. This was achieved by incubating the sections in the post-coupling reaction buffer (1mg/ml Fast Garnet GBC salt, 0.1M sodium acetate buffer pH 6.2) at 4°C for 30 min. The sections were then air dried and mounted in 90% glycerol, 10% PBS.

MOLECULAR BIOLOGY METHODS

II.2 RNA extraction and manipulation

Sterile disposable plasticware was used throughout the preparation of RNA. As general laboratory glassware was often a source of RNAse contamination, it was treated with a solution of 0.1% diethyl pyrocarbonate (DEPC) for 12 hours at room temperature and then autoclaved. DEPC is a strong inhibitor of RNAses, but it is necessary to remove all traces of this compound after treatment as it may inactivate RNA by carboxymethylation. Similarly, solutions used for the preparation of RNA were treated with 0.1% DEPC overnight and then autoclaved. Gloves were worn during all stages of the preparation of RNA, as RNAses are secreted on the surface of hands.

II.2(i) RNA purification

Total RNA was prepared from monolayer cell cultures by a microscale method based on that of Chomininski and Sachi, (1987).

Following treatment, the medium was removed from the cells and stored at -20°C for bioassay. 500ul of denaturing guanidine thiocyanate solution (4M guanidinium

isothiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1M 2-mercaptoethanol) was added to the petri dishes and the cells harvested with a cell scraper. The following solutions were then added sequentially with mixing between each addition : 50ul 2M sodium acetate pH 4, 500ul phenol (water-saturated), 100ul chloroform/ isoamyl alcohol (49:1). Tubes were vigorously shaken for 10 seconds and then cooled on ice for 15 min. The mixture was then centrifuged at high speed in a microfuge for 10 min to form two layers. The top aqueous layer which contained the RNA was removed and placed into a new tube.

The RNA containing layer was extracted twice with an equal volume of chloroform /isoamyl alcohol to remove any traces of phenol. The RNA was precipitated with an equal volume of isopropanol at -20°C overnight. The precipitated RNA was pelleted in a bench microfuge at 15000 for 10 min, and then resuspended in 150ul of solution D. The RNA was reprecipitated using an equal volume of isopropanol at -20°C for at least 1 hour. Again the RNA was pelleted using a bench microcentrifuge. The pellet was washed twice in 200ul of 70% ethanol, followed by a single wash in 100% ethanol, and then dried in a vacuum desiccator. The RNA pellet was then resuspended in 50ul of DEPC-treated sterile water. The amount of RNA purified was quantified by measuring the absorbance at 260nm and 280 nm of a 1 in 25 dilution of the prepared RNA. An absorbance value of 1.0 corresponds to RNA at a concentration of 40ug/ml. Typical yields were 50-100ug/10^6 cells. The RNA was then loaded onto Northern blots or slot blots for analysis.

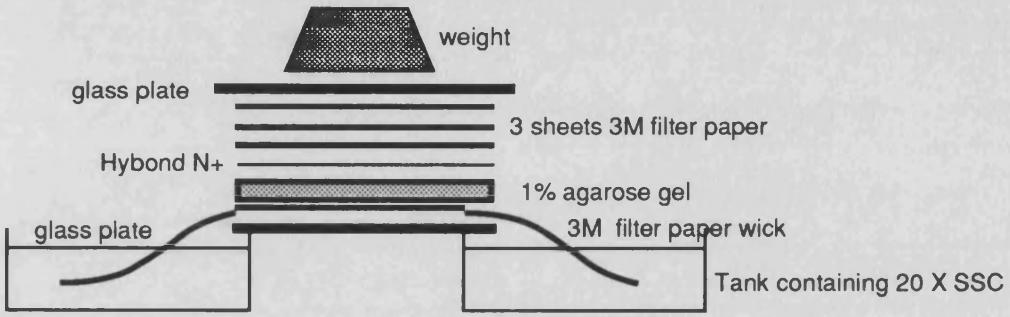
II.2(ii) Northern blotting

Northern blot analysis was carried out according to (Goldberg et al.1979).

Ten microgram aliquots of RNA were precipitated using an equal volume of isopropanol overnight at -20°C. The pellets were washed twice in 70% ethanol, and once in 100% ethanol and dried in a vacuum desiccator. The dry pellets containing 10ug of total RNA were dissolved in 10ul of loading buffer (1X MOPS, 50% (v/v)

formamide, 17% (v/v) formaldehyde, 23% (v/v) DEPC water). The samples were denatured at 65°C for 5 min to remove any secondary structure, and quenched on ice prior to loading. 2ul of RNA loading dye (10 X = 20% (w/v) Ficoll, 0.25% (w/v) bromophenol in 10mM Tris pH 7.8) was then added, and the RNA loaded onto a 1.2% (w/v) agarose denaturing gel in MOPS buffer (10 X MOPS = 0.4M MOPS pH 7.0, 100mM sodium acetate, 10mM EDTA).

The gel was run at 30mA for 5 hours until the dye front was approximately 5 cm from the end of the gel. The gel was then cut to remove the lane with ribosomal markers. This portion of the gel was stained with 0.01% ethidium bromide solution to visualize the 28s and 18s ribosomal bands. The Northern blot was then assembled as shown.



The blot was left overnight for the RNA to transfer to the Hybond N^+ membrane. The RNA was fixed, by placing the membrane on a pad of Whatman 3M paper containing 50mM sodium hydroxide solution for exactly 5 minutes. The blot was then washed in 2 X SSC to remove any traces of sodium hydroxide. The membranes were stored wrapped in Saran Wrap at 4°C.

II.2(iii) Slot blotting

The slot blot method used purified RNA prepared by a microscale method based on that of Chomininski and Sachi, (1987).

Ten microgram aliquots of RNA were mixed with an equal volume of formamide, denatured at 65°C for five minutes and then quenched on ice. The salt concentration was adjusted to 0.25 M with sodium acetate. Two-fold serial dilutions were then made and loaded in 100ul volume onto a GIBCO-BRL slot blot manifold. The samples were

washed through with a solution of 50% formamide/0.25M sodium acetate pH 5.2. The RNA was then fixed to the filter with a solution of 50mM sodium hydroxide for 5 minutes. The filter was finally washed in 2 X SSC and stored at 4°C wrapped in Saran wrap.

II.2(iv) Quantification of RNA on Northern and Dot blots

Blots were stained with 2% methylene blue, and then destained in 2 X SSPE (20 X SSPE = 3M sodium chloride, 0.2M sodium dihydrogenphosphate, 0.02M EDTA) prior to hybridization to check for equal loading. After hybridization with the cytokine cDNA probe, the blots were stripped and rehybridized with the housekeeping gene β-actin. β-actin is thought to be a constitutive non-modulated gene, and therefore the hybridization signal should be the same for all the samples. The slots and bands were scanned with a hand held densitometer (Genetic Research Inc) for quantification. The readings were adjusted for small variations in the amount of RNA in each slot/track by normalizing with respect to the β-actin value.

II.2(v) Stripping radioactive probes from blots

The radioactive probe was removed from the Northern blots or slot blots by boiling the filter in a solution of 0.1% SDS. After cooling to room temperature and washing in 2 X SSC, the filters were exposed to film to check that all the probe had been removed.

II.2(vi) Polyacrylamide gel electrophoresis of RNA probes

The sizes of the RNA probes were determined by polyacrylamide TBE vertical gel electrophoresis. The gel was prepared by adding 21g of urea to a polyacrylamide gel solution (3.5% acrylamide [29:1 acrylamide:bis]) and warming the solution to 37°C until the urea dissolved. Once the urea was in solution, 100ul of TEMED and 160 ul of 16% ammonium persulphate were added to initiate and catalyse the polymerization of the gel solution respectively. The gel solution was then mixed gently and poured onto a

clean glass electrophoresis plate which had 0.5 mm spacers fixed to its edges. As the gel was poured, a second sialanized gel plate was moved across the surface of the bottom gel plate to form a "gel sandwich". It was necessary to carry out this procedure rapidly to avoid the gel solution polymerizing before the gel was prepared. A 0.5 mm comb was inserted into the top of the gel to form sample wells. The gel was left at room temperature for a hour to fully polymerize, afterwhich the comb was removed and the wells rinsed with water. The gel was then attached to the electrophoresis apparatus and the reservoirs filled with 1 X TBE running buffer.

The RNA samples were dissolved in 1 X TBE, denatured at 65°C and quenched on ice and loading buffer (30% Ficoll, 0.25% bromophenol blue, 0.25% xylene cyanol, 0.2M EDTA pH 8.0) was added at a 1 in 10 dilution. The RNA samples were loaded into the sample wells using an ultra thin pipette tip. DNA standards from a sequencing reaction were electrophoresed alongside the samples for accurate sizing. The gel was run at 30mA and the voltage monitored to prohibit it rising over 1500V, electrophoresis was stopped when the bromophenol blue dye front was two thirds down the gel. The RNA was fixed in position by immersing the gel in 10% methanol, 10% acetic acid for 30 min. The gel was then carefully mounted on Whatman 3M paper and dried down for 1 hour at 70°C. The dried gel was then autoradiographed for 24 hours at room temperature.

II.3.DNA purification and manipulation

II.3(i) Preparation of competent cells

A single colony of the host strain bacteria was isolated from the frozen stock by streaking the bacteria onto an agar plate with appropriate antibiotic selection. The single colony was used to innoculate a 5ml culture in L-broth (5g yeast extract, 20g bactotryptone, 5g sodium chloride/litre of medium), which was then incubated overnight at 37°C. A subculture was prepared by diluting the 5 ml aliquot 1 in 20 in

L-broth. The bacteria were cultured for approximately 2 further hours until the optical density of the solution was 0.3 at 600 nm. The flask was quickly chilled on ice to arrest bacterial growth, and the bacteria were then spun down into a pellet in a 50 ml polypropylene tube at 6000 r.p.m.for 5 min at 4°C. The bacterial pellet was gently resuspended in a 2/5 volume transformation buffer I (30mM potassium acetate, 100mM $RbCl_2$, 10mM $CaCl_2$, 50mM $MnCl_2$, 15% (v/v) glycerol pH to 5.8). The bacteria were then left on ice for 5 min then pelleted again at 6000 r.p.m 5 min at 4°C. Following this the pellet was resuspended in a 1/25 volume of transformation buffer II (10mM MOPS, 75mM $CaCl_2$, 10mM $RbCl_2$, 15%(v/v) glycerol, pH 6.5). The bacteria were then left on ice for 15 min during which time the bacterial cell wall modifies to give the bacteria a spheroplast morphology. The bacteria could then either be transformed immediately, or aliquots of 200ul placed into prechilled freezing vials and snap frozen in dry ice. The aliquots were stored liquid nitrogen and used within 6 months. The competency of the bacteria was tested by transforming 10 ng of pUC 19. The transformation efficiency was usually in the order of 10^8 colonies/ng DNA.

II.3(ii) Transformation of competent bacteria.

The aliquot of competent bacteria was thawed at room temperature and stored on ice for 10 min. The plasmid DNA (10ng-100ng) was then added directly to 200ul of competent bacteria. This mixture was then left on ice for 15 - 45 min so that the DNA could become associated with the bacteria. The bacteria were then "heat-shocked" at 42°C for 2 min and returned to ice for 1-2 min. Two volumes of L-broth were then added at room temperature and the tube incubated at 37°C for 60 min with gentle shaking. This process allowed the bacteria to recover. It was important that no more than 2 volumes of L-broth were added as this is thought to promote bacterial division. An aliquot of bacteria (typically 10 ul) was then spread onto agar plates containing 100ug/ml ampicillin. The plate was incubated at 37°C overnight.

II.3(iii) Micropreparation of DNA (Minipreps)

Small amounts of plasmid DNA for restriction enzyme analysis were prepared by a modified method of Birnboim and Doly, (1979).

A plasmid colony was grown overnight in 10 ml of L-broth containing 50ug/ml ampicillin. 4.5 ml of the culture was spun down in a 1.5ml microfuge tube, by repeatly adding the bacteria culture medium and removing the supernatant. The bacterial pellet was resuspended in 100ul of ice cold Solution I (50mM glucose, 25mM Tris pH 8.0, 10mM EDTA, 10ug/ml lysozyme) by vigorously vortexing the tube. 200ul of freshly prepared Solution II (0.2M sodium hydroxide, 1% SDS) was added to the suspension and the tube gently mixed, the samples were left on ice for 30 min to ensure bacterial lysis. 150 ul of ice-cold solution III (3M potassium acetate, 5M acetic acid) was mixed with the highly viscous lysate, the tubes were left on ice for 30 min to precipitate protein. The protein appeared as a white flocuant precipitate, which was removed from the supernatant by spinning the lysate in a microfuge. Residual protein was removed by phenol extraction of the supernatant followed by 2 chloroform extractions to remove traces of phenol. The plasmid DNA was then precipitated with 2 volumes of ethanol at 20°C for 1 hour. The plasmid DNA was pelleted in a microfuge for 5 min, washed twice in 70% ethanol and once in 100% ethanol and then dried in a vacuum dessicator. The DNA was finally dissolved in sterile water. Typical yields attained, were 3 - 5 ug per 4.5ml bacterial culture. 2ug of plasmid DNA was then used for restriction enzyme analysis.

II.3(iv) Large scale plasmid purification (Maxipreps)

When more than 100ug of plasmid DNA was required, a QIAGEN Maxi prep kit was used. Ion exchange columns in this kit bind DNA preferentially and allow its purification from the bacterial lysate.

The following procedure was used: 200ml of bacterial culture was incubated on a shaking platform at 37°C overnight, or until the broth was turbid with bacterial growth. The bacteria were harvested in a high speed centrifuge at 3000 rpm for 15 min and the resulting bacterial pellet was resuspended in 4.0 ml of buffer P1 (50mM Tris pH 8.0, 10mM EDTA, 400ug RNase A). The bacteria were lysed with 4.0 ml of buffer P2 (200mM sodium hydroxide, 1% SDS) which was gently added and the solution incubated at room temperature for 5 min. 4.0 ml of buffer P3 (2.55 M potassium acetate) was added to neutralize the lysate. The lysate was then centrifuged at 5000 rpm and 4°C for 30 min to pellet the bacterial protein. The resulting supernatant was loaded onto a QIAGEN midi column that had been pre-equilibriated with buffer QB (750mM sodium chloride, 50mM MOPS pH 7.0, 15% ethanol). The column was then washed with 8.0 ml of buffer QC (1000 mM sodium chloride, 50 mM MOPS pH 7.0, 15% ethanol) to remove any impurites which may have bound. The DNA was eluted with 2.0 ml of buffer QF (1200 mM sodium chloride, 50 mM MOPS pH 8.0, 15% ethanol), and then precipitated with 0.8 volumes of isopropanol at room temperature for 5 min. The precipitate was harvested by centrifugation at 13,000 r.p.m./4°C for 30 min. The pellet was washed twice with 200ul of 70% ethanol, once with 100% ethanol and dried in a vacuum dessicator. The DNA was dissolved in sterile water and a 1 in 100 dilution measured at 260/280 nm in a spectrophotometer (A_{260} = 1.0 = 50ug/ml of double stranded DNA). Typical yields obtained, were 150 ug/200ml culture of a medium copy number plasmid.

II.3(v) Restriction digests of plasmid DNA

Restriction fragments containing cDNA sequences for the cytokines IL-1β and TGFβ, were prepared from plasmid constructs. These fragments were purified as probes for the hybridization of RNA blots.

Both IL-1β and TGFβ cDNAs were 300 bp fragments cloned into pGEM plasmids. The IL-1β insert was liberated by digesting 10ug of pGEM IL-1 with 20 units of both

PstI and EcoRI in High buffer (Boeringer Manheim) for 1 hour at 37°C. The TGFβ insert was prepared by digesting 10 ug of PGEM TGF in Medium buffer (Boeringer Manheim) with 20 units of both Hind III and EcoRI under the same conditions. The inserts were then separated by gel electrophoresis, purified by the Geneclean method and stored at - 20°C until required for labelling. The osteopontin cDNA was a 1.2 kb fragment in Bluescript.

RNA probes were generated for *in situ* hybridization using linearized plasmid as the template. Sense and antisense transcripts were generated using the Manheim Boeringer *In vitro* transcription probe kit, from linear templates prepared using restriction enzymes at the 5' and 3'ends of the inserts. The specific activity of the probes were routinely in excess of 10^8cpm/ug.

II.3(vi) Electrophoresis of DNA

DNA fragments were separated by electrophoresis on a "submarine" 1% (w/v) agarose gel in TAE buffer (10 X TAE = 0.4M Tris, 0.05M sodium acetate, 0.01M EDTA pH 7.8) containing 0.5ug/ml ethidium bromide. The DNA was loaded in a solution containing a 1/10 volume loading buffer (20% w/v Ficoll, 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol in 10mM Tris pH 7.8, 10mM EDTA). Usually 1ug of DNA was loaded per lane in diagnostic gels. The gels were run at approximately 60mA, until good separation was achieved. The DNA bands were visualized using a transilluminator emiting uv light. The gel was photographed using Polaroid Type 55 positive/negative film.

II.3(vii) Extraction of DNA fragments from agarose gels

The DNA was purified from the agarose using a Geneclean kit from Stratech Scientific Ltd. The principle of this kit is that the DNA fragments bind to glass milk beads once released from the dissolved agarose. The DNA can then be eluted directly from the beads so avoiding the time-consuming ethanol precipitation step.

The DNA was electrophoresed on a 1% agarose gel, and the band excised using a scapel blade. The slice of gel was then weighed and 2 - 3 times the equivalent weight of saturated sodium iodide added, and the agarose melted at 55°C for 5-10 min. 5ul of the glass milk beads (for < 5ug of DNA) were added and the tube mixed before leaving at room temperature for 15 min to enable the DNA to bind. The tube was then centrifuged for 5 sec and the supernatant removed and saved until the technique was completed. The glass milk pellet was washed 3 times in Newash. After the final wash all traces of Newash were removed with a fine pipette tip. The DNA was eluted by adding 10 ul of sterile DEPC water, heating to 55°C for 5 min before spinning for 30 sec and removing the supernatant. This elution step was repeated, and the supernatants combined. The yield obtained was typically between 70 - 80%.

II.4. Hybridization techniques

II.4(i) Radiolabeling probes

The cDNA probes were radiolabelled using the random hexanucleotide priming reaction supplied as an Amersham kit. The specific activity was monitored by taking 1ul from the final 50ul volume and "spotting" it onto two DE18 filter papers which bind polynucleotides. After drying, one of the filter papers was washed 3 times in 0.5M sodium dihydrogen phosphate solution to remove any unincorporated radioactive nucleotides. Both filters were then scintillation counted. The specific activity of the probes were then calculated by expressing the counts on the washed filter as a percentage of those of the unwashed filter. The probes were routinely in excess of 40% incorporation (10^7cpm/ug).

II.4(ii) Northern blot /slot blot hybridization

The hybridization step was carried out at 52°C as described by Goldberg et al (1979), using a solution containing 33% formamide, 2 X SSC, 10% dextran sulphate, 100 ug/ml ssDNA and 100mM EDTA .

Post-hybridization washes were carried out using 2 X SSC at room temperature, followed by 2 X SSC + 0.1% SDS at 65°C. If greater stringency was required, a final wash in 0.2 X SSC at room temperature was performed. Autoradiographs were established by exposing the filter to X-ray film for 24-72 hours at -70°C with an intensifying screen.

4 (iii) *In situ* hybridization

The tissues for *in situ* hybridization were "snap" frozen in a dry ice ethanol bath in a 5% solution of PVA immediately after surgery, and then stored at -70°C for up to 3 months prior to sectioning. The tissue sections were cut on a Bright cryostat, and mounted on four well multi spot slides which had previously been treated with TESPA to improve adherance of the sections. The sections were fixed with 4% paraformaldehyde/PBS for 5 minutes and then washed with PBS to remove any traces of paraformaldehyde. The slides were dehydrated through an ethanol series and stored for up to four weeks at -20°C. The following pretreatments were performed to decrease the amount of non-specific binding of the riboprobes to the specimen: using a demineralisation 0.25M sodium citrate buffer pH 4.5 for 1 hour at room temperature (found to be vital for removing non-specific binding of the probe to the bone matrix). Proteinase K (1ug/ml) treatment at 37°C for 30 min removed a proportion of the structural proteins and the remaining proteins were acetylated with acetic anhydride (0.1M at room temp. for 10 min). Finally a prehybridization in 2 X SSC /50% formamide was performed at room teperature for at least 10 min.

The RNA probes were synthesized using SP6 and T7 RNA polymerases according to the Manheim Boeringer transcription kit. The specific activity of the riboprobes were in excess of 10^8cpm/ug. Sense transcripts were synthesized to act as a negative control and assess non-specific binding.

The hybridization was carried out in a solution containing 10% dextran sulphate, 50% formamide, 10mM DTT, 2 X SSC, 200ug/ml ssDNA, 1 X Denharts, 50ug/ml tRNA,

100 U/ml RNAsin at 45°C overnight. The post-hybridization washes were as follows: 4x15mins 2 x SSC at room temperature, 4 X 15mins 2 x SSC at 37°C, RNAse A (200ug/ml) in 0.1M Tris/ 50mM EDTA pH 8.0 at 37°C for 30 min. Finally a high stringency wash in 0.2 X SSC at 37°C for 30 min was performed to remove any residual non specific binding. After washing, the slides were dehydrated through an ethanol series, air-dried and dipped in LM-1 photographic emulsion. The slides were exposed at 4°C for up to one week prior to developing.

Chapter III : The expression of transforming growth factor beta by human osteoblast-like cells *in vitro*.

III.1 ABSTRACT

Transforming growth factor beta (TGFβ) is one of the most potent osteotropic cytokines and is deposited in large amounts in bone matrix. For these reasons the expression of TGFβ mRNA was studied in human osteoblast-like cells *in vitro*. These cells constitutively expressed TGFβ1, as determined by both *in situ* hybridization and Northern blot analysis. Immunolocalization studies confirmed that a population of osteoblasts expressed TGFβ protein both constitutively and after stimulation with 1,25-dihydroxyvitamin D_3 (1,25 D_3). Treatment of the cells with the systemic hormones 1,25 D_3 (10^{-8}M) and parathyroid hormone (10^{-7}M) increased TGFβ mRNA expression by 3 fold and 9 fold respectively. In contrast, treatment with retinoic acid (10^{-8}M) an osteoblast maturation factor had no effect on TGFβ mRNA expression. Treatment of the cells with hydrocortisone (10^{-8}M) resulted in the complete suppression of TGFβ1 mRNA expression. This inhibition was partially alleviated by the addition of 1,25 D_3 (10^{-8}M), and the expression of TGFβ1 mRNA returned to 70% of the stimulated level. Physiological doses (10^{-9} - 10^{-10} M) of 17-β oestradiol resulted in a 5 fold increase in TGFβ1 mRNA expression, further confirming the importance of systemic hormones as modulating agents of TGFβ mRNA expression.

The cytokines IL-1α (200pg/ml), TNFα (17ng/ml), and the bacterial lipopolysaccharide (LPS) (500ng/ml) failed to stimulate TGFβ mRNA expression. In contrast, TGFβ1 treatment (100pM) resulted in an autoinductive stimulation of TGFβ1 mRNA expression.

In summary, TGFβ expression was found to be modulated by the systemic hormones but not by the "inflammatory" cytokines.

III.2 INTRODUCTION

TGFβ is a 25 kDa homodimeric polypeptide first identified by its ability to cause a phenotypic transformation of rat fibroblasts (Roberts et al. 1981). It is expressed by virtually every cell type and has been shown to have many regulatory actions, including the modulation of proliferation, differentiation and protein expression (Roberts et al. 1985). This cytokine is secreted in a latent form as a 390 amino acid precursor molecule which must be cleaved to form an active 112 amino acid monomer. A family of at least 5 different highly homologous isoforms (termed TGFβ 1 - 5) have been identified by screening cDNA libraries with TGFβ1 probes. A high degree of conservation (99%), between human and mouse $TGF\beta_1$ sequences argues for a critical biological role of TGFβ across species.

There has been much interest in the role of TGFβ in bone cell biology, since it has been found to be deposited in the bone matrix in large amounts (Gehron Robey et al. 1987). Initially two isoforms of TGFβ, now referred to as $TGF\beta_1$ and $TGF\beta_2$, were identified. The former was first isolated from human platelets and was found to be identical to CIF A, a cartilage inducing factor (Ellingsworth et al. 1986). Both forms are present in bone matrix (Celeste et al. 1990), although $TGF\beta_1$ is four times as abundant as $TGF\beta_2$. They are highly homologous with over 70% identity at the protein level.

The results obtained from many studies of the action of TGFβ on bone cells *in vitro* are complex and in some cases conflicting. The consensus of opinion however is that TGFβ may "couple" bone formation to bone resorption in the bone remodelling cycle. TGFβ is a very attractive candidate as a coupling factor for many reasons, which are summarised in figure III.1. As mentioned earlier TGFβ is deposited in the bone matrix in a latent form which must be activated. This can be achieved *in vitro* by limited proteolysis or acidification. In the bone matrix it is thought that TGFβ may be activated by osteoclasts. There is some evidence to support this idea, since Oreffo et al. (1989) demonstrated that isolated osteoclasts are able to activate latent TGFβ, presumably in the acidic microenvironment under their ruffled border. A variety of

osteoblastic cell lines, marrow fibroblasts and monocytes were unable to activate latent TGFβ suggesting this is a highly specific property.

After a period of resorption it is necessary for the quantity of bone that has been resorbed to be replaced. This requires the availability of large numbers of osteoblasts. TGFβ may increase the availability of osteoblasts in several ways. Firstly, it may act as a recruitment factor to increase the number of osteoblasts present at the remodelling site, since it has been shown to be chemotactic for these cells *in vitro* (Pfeilschifter et al. 1990). Secondly, it has been postulated that TGFβ may increase osteoblast numbers as it is a potent stimulator of osteoblast proliferation *in vitro*, although this seems to be a biphasic effect and dependent on cell density (Centrella et al. 1987).

Bone formation obviously involves the accelerated synthesis of the bone matrix by osteoblasts. TGFβ is thought to be important in promoting the synthesis of extracellular matrix. It has been shown to upregulate RNA transcription and translation of many matrix components including type I collagen, fibronectin (Ignotz and Massague 1986) and osteonectin (Thiebaud et Al.1990), resulting in an increase in matrix deposition of these proteins. In addition to promoting the synthesis of matrix proteins, there is evidence to suggest that TGFβ may also have a protective role over the bone matrix. Studies with mouse fetal calvarial cells show that TGFβ increases the synthesis of TIMP (tissue inhibitor of metalloproteinases), an inhibitor of type I collagenase and stromelysin, two enzymes which can degrade the extracellular matrix (Meikle et al. 1991).

The role of TGFβ in bone resorption remains controversial since it has been shown to inhibit (Pfeilschifter et al. 1988) and promote (Tashjian et al. 1985) bone resorption in different model systems (see chapter I). Most of the data suggests that TGFβ has a largely anabolic effect in bone, since both TGFβ1 and TGFβ2 abolish both 1,25 D_3 and IL-1 induced resorption in rat long bone to some degree (Pfeilschifter et al. 1988). It is possible that the stimulation of bone resorption seen in the mouse calvarial model is a non specific prostaglandin mediated effect. Further evidence that TGFβ inhibits

resorption comes from studies on isolated osteoclasts, where both isoforms of TGFβ decrease TRAP activity and oxygen free radical generation (Oreffo et al. 1990).

During the quiescent stage of bone remodelling TGFβ which has been deposited in the matrix by osteoblasts remains bound to matrix proteins. Subsequently it is liberated at the initiation of another round of remodelling. In summary, TGFβ is thought to have a pivotal role in bone remodelling since it may provide the mechanism for the coupling of resorption to formation.

The biological effects of TGFβ on bone have been well documented, but there is very little information about the regulation of TGFβ expression in the osteoblast. We therefore undertook this study to investigate the modulation of TGFβ expression in osteoblasts. Since we wanted to assess the results with reference to human disease states it was necessary to use human cells, as the effects of many growth factors and cytokines on bone cells have been shown to be species specific. For example TGFβ has been implicated as having an effect on osteoclast formation: at low concentrations it inhibits the formation of osteoclasts from long term human marrow cultures (Chenu et al. 1987) but promotes their formation in mouse cultures (Shinar and Rodan. 1990).

It was considered to be very important to use primary human osteoblast cultures since the popular alternative osteoblastic cell lines derived from osteosarcomas do not express the same phenotype as normal osteoblasts in culture (Clover and Rickard, unpublished results). Therefore human bone cells were obtained by culture from explants of bone, and treated with a variety of cytokines and systemic hormones to determine their effects on TGFβ mRNA expression. Northern blot analysis was used to monitor TGFβ expression as this is a very sensitive method and is superior in reliability and reproducibility to bioassays.

Figure III.1. Possible roles for TGFβ in the bone remodelling cycle.

III.3 METHODS AND MATERIALS

Northern and slot/dot blots

Human bone cells were obtained from explants of bone (as described in chapter II.1(i)). After 4 - 6 weeks the osteoblast-like cells were confluent in the $9cm^2$ petri dishes. Since each petri dish did not contain exactly the same population of osteoblast-like cells, it was necessary to pool the cells for the different experiments. For the Northern and slot blot experiments 10 -12 petri dishes of osteoblast-like cells were prepared as follows. Firstly a short wash in PBS was undertaken to remove trypsin inhibitors, then the cells were treated with trypsin (50U/ml) at 37°C for 5 mins until all the cells were detached from the tissue culture plate. The trypsinization reaction was then stopped by the addition of medium containg 10% fetal calf serum (FCS). The cells were harvested into a sterile tube at 1000 rpm in a bench cenrtifuge for 5 min; the resulting pellet was then washed twice in medium (1xMEM, 10% FCS, 1mM glutamine, 100U/ml penicillin /streptomyocin pH 7.2). The cells were counted and seeded into $9cm^2$ petri dishes at a density of 0.5 - 1 million per dish, and left overnight in a 10% CO_2 atmosphere at 37°C to allow the cells to recover and adhere. The following day the cells were washed twice with PBS to remove any serum, then fed with a synthetic serum-free medium (1xMEM, 0.1% BSA, 10ug/ml transferrin, 1ug/ml insulin, $3x10^{-8}M$ sodium selenate, 1mM glutamine, 100U/ml penicillin /streptomyocin). After a further 24 hours the cells were treated with the agents. After treatment the medium was removed from the petri dishes, and the RNA harvested by adding 0.5 ml of denaturing guanidine hydrochloride solution, and scraping the petri dish until all the cells were lysed. The RNA was then purified according to a method based on that of Chomininski et al. (1987) (see Chapter II 2(i)). Routinely 1 petri dish was used per treatment with a yield of approximately 30 - 50 ug RNA /$1x10^{-6}$ cells.

The Northern blots and slot/dot blots were carried out as described in chapter II.2(ii), II.2(iii). Ten micrograms of total cellular RNA was used per lane for Northern blots, and 5ug for the highest concentration in slot blots. All filters were stained with

following day the cells were washed twice with PBS to remove any serum, then fed with a synthetic serum-free medium (1xMEM, 0.1% BSA, 10ug/ml transferrin, 1ug/ml insulin, $3x10^{-8}$M sodium selenate, 1mM glutamine, 100U/ml penicillin /streptomyocin). After a further 24 hours the cells were treated with the agents. After treatment the medium was removed from the petri dishes, and the RNA harvested by adding 0.5 ml of denaturing guanidine hydrochloride solution (see chapter II), and scraping the petri dish until all the cells were lysed. The RNA was then purified according to a method based on that of Chomininski et al. (1987) (see Chapter II 2(i)). Routinely 1 petri dish was used per treatment with a yield of approximately 30 - 50 ug RNA /$1x10^{-6}$ cells.

The Northern blots and slot/dot blots were carried out as described in chapter II.2(ii), II.2(iii). Ten micrograms of total cellular RNA was used per lane for Northern blots, and 5ug for the highest concentration in slot blots. All filters were stained with methylene blue (see chapter II 2(iv)) to check for equal loading. The hybridization was carried out according to a method based on that of Goldberg et al. (1979) (chapter II 4(ii)). After post hybridization washing the filters were exposed to X ray film for 1 - 3 days.

In situ hybridization

Human osteoblast-like cells were trypsinized as described above and seeded at a density of 3000 /spot on multiwell slides for *in situ* hybridization. After 24 hours the cells were treated with 1, 25 D_3 or incubated with medium for a further 12 hours, then washed with PBS and fixed in 4% paraformaldehyde/PBS for 5 mins. The slides were then dehydrated through an ethanol series and stored for up to 1 month at -20°C prior to hybridization. The TGFβ RNA probe was generated from the linearized pGHTGFB-27 plasmid which contained a 300 base pair region of the cDNA. Sense and antisense transcripts were prepared using SP6 and T7 RNA polymerase promoters (see chapter VI.3). The specific activity of the RNA probes was checked to prove they

were comparable (chapter II.4(i)). The length of both probes were verified by vertical polyacrylamide gel electrophoresis (chapter II 2(vi)) to check that the majority of the transcripts were of full length. The *in situ* hybridization of the TGFβ riboprobe was carried out as described in chapter II 4(iii) with the following modifications: the demineralisation step was omitted as there is no mineral present in these cultures, and the proteinase K treatment was shortened to 5 mins as the cells contained far less protein than the tissue sections.

Immunolocalization

For immunolocalization studies the cells were stained using a murine TGFβ monoclonal antibody (as described in chapter II.1(iv)).

III.4 RESULTS

Northern blot analysis demonstrated that human osteoblast-like cells were capable of expressing TGFβ mRNA in culture. Figure III.2 shows the expression by osteoblast-like cells from 6 different donors. Although the amount of TGFβ expression varied according to the donor, mRNA was detected in every population of bone cells studied (>30 donors). Two mRNA species were usually detected, the predominant species being a 2.5 kb transcript encoding for TGFβ1, a second 4.5 kb species encoding $TGF\beta_2$ was also detected due to the cross reactivity of the $TGF\beta_1$ probe. In certain cases a third mRNA species of approximately 7.2 kb encoding for $TGF\beta_3$ (see fig III.5) was detected in the bone cell populations, but this was very unusual.

The expression of $TGF\beta_1$ by human bone cells was confirmed by *in situ* hybridization studies. Fig III.3 shows that the entire population of human osteoblast-like cells constitutively expressed $TGF\beta_1$ mRNA in culture. Since the presence of $TGF\beta_1$ mRNA does not confirm the synthesis of TGFβ1 protein, immunolocalization studies were undertaken to confirm the expression of TGFβ at the protein level. Figure III.4 shows a population of human osteoblast-like cells expressing TGFβ protein. It must be noted that approximately 20% of the cells seemed to be expressing the protein in comparison to the entire population expressing the TGFβ mRNA. Figure III.4b shows a high magnification photograph of a human osteoblast-like cell expressing $TGF\beta_1$. The staining can be seen to be perinuclear, which is typical for a secretory protein whose secretion has been blocked at the Golgi apparatus.

After the confirmation that human osteoblast-like cells were capable of producing TGFβ, a study was undertaken to determine which cytokines and osteotropic factors modulated the expression of TGFβ mRNA in these cells. The systemic hormone 1,25 D_3 had been shown previously to increase the amount of TGFβ activity in tissue culture supernatants from rat osteoblast-like cells in culture (Pfeilschifter and Mundy, 1987). Therefore the human osteoblast like cells were treated with 1,25 D_3 at 10^{-8}M a concentration which had previously been shown to induce alkaline phosphatase activity

in these cells. Figure III.5 shows that 1,25 D_3 treatment gives rise to an increase in TGFβ_1 mRNA expression, the induction was fairly small (approximately 3 fold), but highly reproducible (5 out of 6 donors). The increase in TGFβ mRNA was detectable after six hours in most cases, with expression reaching a peak at eight hours, after 12 hours the level decreased to below basal expression (Fig III.6). There was some variability in the time courses for the 1,25 D_3 stimulation. The overall trend in cells from different donors was similar, but in some cells the mRNA levels took longer to peak and longer to return to a basal expression. Cells were treated with 1,25 D_3 for 48 hours to monitor the effect of this agent on protein synthesis, however there was no increase in the number of cells expressing TGFβ1 in the population (data not shown).

Retinoic acid (10^{-8}M) is known to act as a differentiating agent in certain osteoblastic cell lines (Heath et al. 1989), therefore its effect on TGFβ mRNA expression was investigated. It had no effect on TGFβ_1 mRNA expression (Fig III.5, lane 2) at concentrations which had previously been shown to affect osteoblast activity (Evans, unpublished observations).

Parathyroid hormone has been shown to be an important factor in modulating the expression of several proteins by osteoblast-like cells. In this study parathyroid hormone was shown to be an effective stimulus for TGFβ_1 mRNA expression. Treatment of cells with 10^{-7}M PTH for six hours resulted in a 9 fold increase in TGFβ_1 mRNA (Fig. III.7). The effect was apparent after six hours, but not sustained, with the mRNA levels returning to control levels after 12 hours. It is interesting to note that both 1,25 D_3 and PTH have a very similar time course of stimulation with an effect being detected after 6 - 8 hours in both cases.

The expression of TGFβ1 mRNA was completely inhibited by treatment with (10^{-7}M) hydrocortisone (Fig. III.8, lane 3). When 1,25 D_3 was added in conjunction with

hydrocortisone the suppression was partially alleviated (Fig III.8, lane 1) and the mRNA level was restored to approximately 70% of the stimulated level.

There is much evidence to suggest that the sex hormone status of an individual influences bone mass, particularly in postmenopausal females (Lacey et al. 1991). Therefore the effect of 17-β oestradiol treatment on TGFβ mRNA was monitored. This agent gave rise to a 6 fold increase in TGFβ mRNA expression after 8 hours, with TGFβ mRNA levels returning to basal expression after 24 hours (Fig. III.9). The modulation showed a biphasic concentration effect at physiological doses, $1x10^{-10}M$ was the most effective concentration in stimulating expression, with $1x10^{-8}M$ giving a slight inhibition of expression (Fig. III.10).

It has been reported that TGFβ is able to autoregulate its own expression (Kim et al. 1990). Cells treated with 100pM TGFβ in serum free culture showed a small increase in TGFβ mRNA expression after six hours (Fig. III.11). The lower dose of 10pM, although capable of stimulating proliferation in these cells (Fig. III.12) did not increase TGFβ expression.

The "inflammatory cytokines " were screened to determine whether they modulated TGFβ mRNA expression in human osteoblast-like cells. Treatment of the cells with IL-1α (200pg/ml) resulted in no increase in $TGF\beta_1$ mRNA, which remained at the basal level (Fig III.13, lane 2). Tumour necrosis factor alpha also did not appear to modulate TGFβ expression over a 24 hour timecourse (Fig III.14). The non specific inducer of inflamatory responses, LPS also did not have any effect on $TGF\beta_1$ mRNA expression (Fig 12 lane 3).

III.4 FIGURES

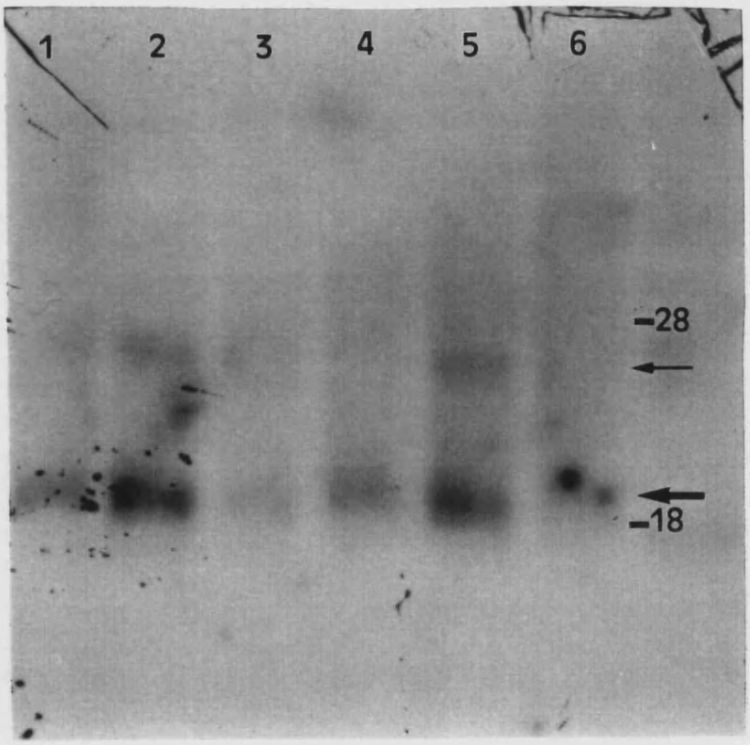
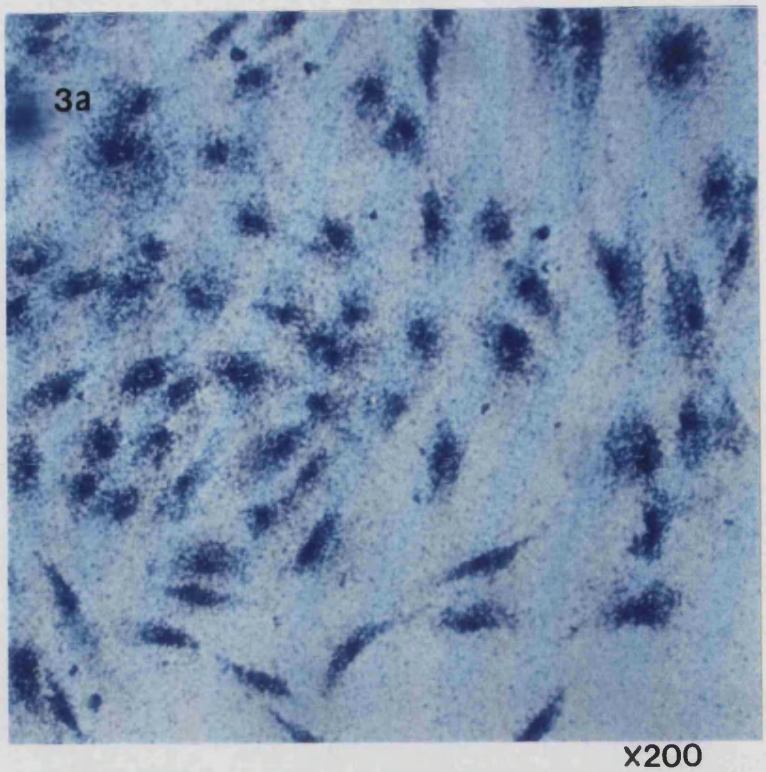
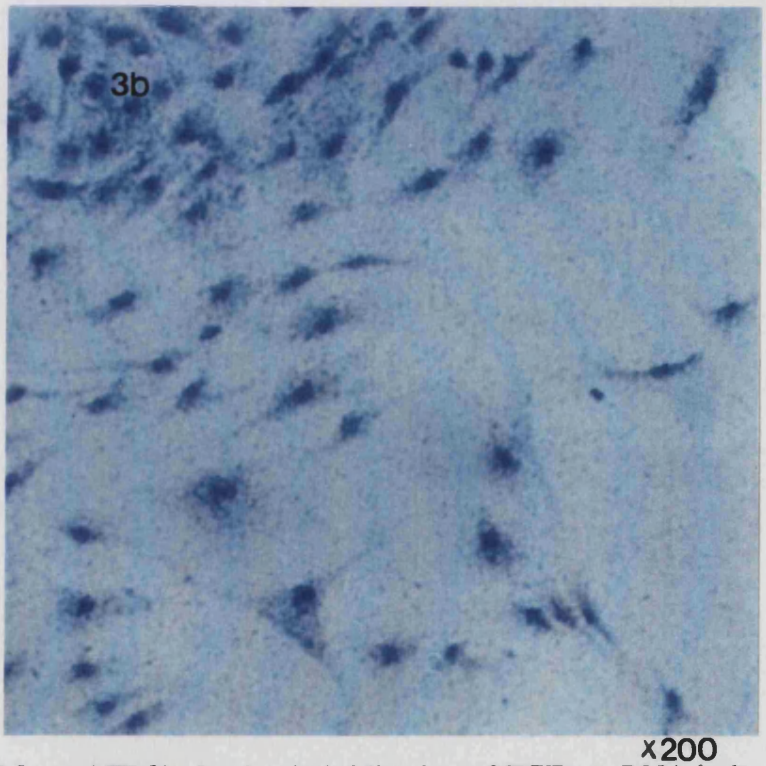


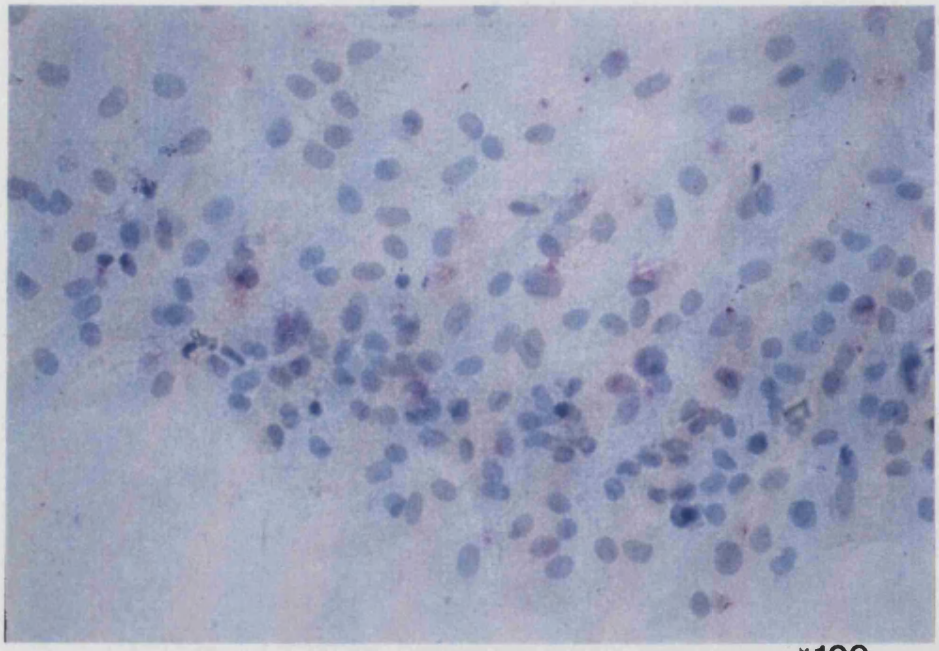
Figure III.2. The constitutive expression of TGFβ mRNA by human osteoblast-like cells in culture. Total RNA was extracted from osteoblast-like cells derived from six different donors (LANES 1-6 (chapter 2). Ten micrograms of total RNA was loaded in each lane of the Northern blot. Two mRNA transcripts are present : the predominant species is 2.5 kb (large arrow) and encodes $TGF\beta_1$; the second transcript of approximately 4.2 kb (small arrow) encodes $TGF\beta_2$. The figures 18 and 28 refer to the positions of the 18s and 28s ribosomal bands. These transcripts are approximately 2 and 4 kb respectively and are used as markers to determine the TGFβ transcript sizes.



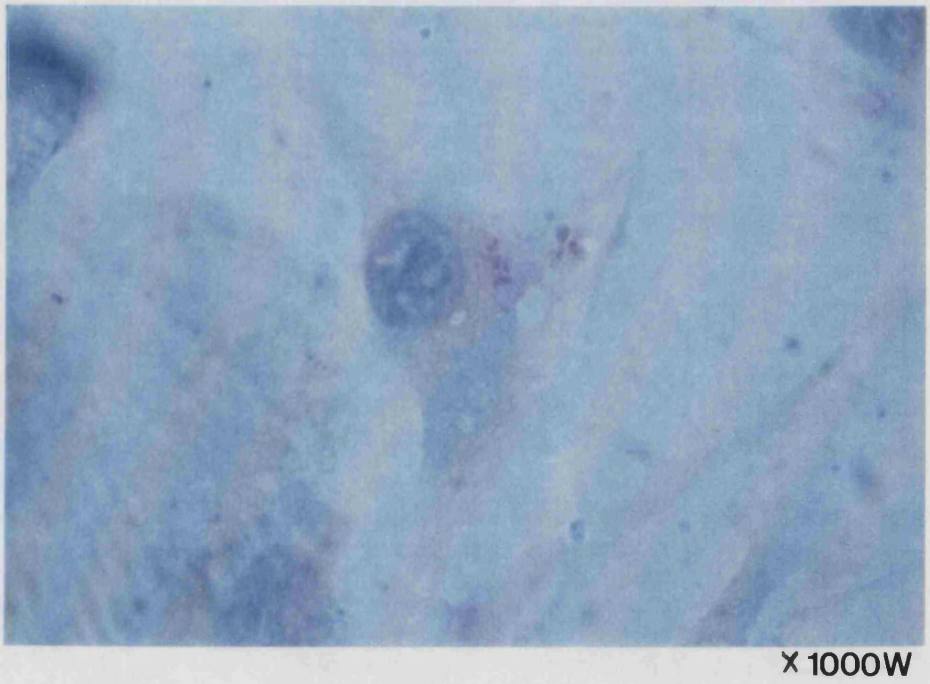


Figures III.3a and III.3b. *In situ* hybridization of TGFβ mRNA in human osteoblast-like cells.

The *in situ* hybridization was carried out as described in Materials and Methods. Grains represent binding of the antisense probe. The entire population of osteoblasts expressed TGFβ mRNA. Figure 3b shows that very little non-specific hybridization is observed with the sense negative control probe.



Figures III.4a and III.4b. Immunolocalization of TGFβ protein in human osteoblast-like cells in culture. Cells were cultured as described in Materials and Methods, and stained for endogenous alkaline phosphatase activity (blue stain). TGFβ expression (pink stain) was detected by using a mouse monoclonal antibody with an alkaline phosphatase conjugated secondary antibody. Figure III.4b is a higher magnification photograph of a single cell and shows the perinuclear staining associated with accumulation in the Golgi apparatus.



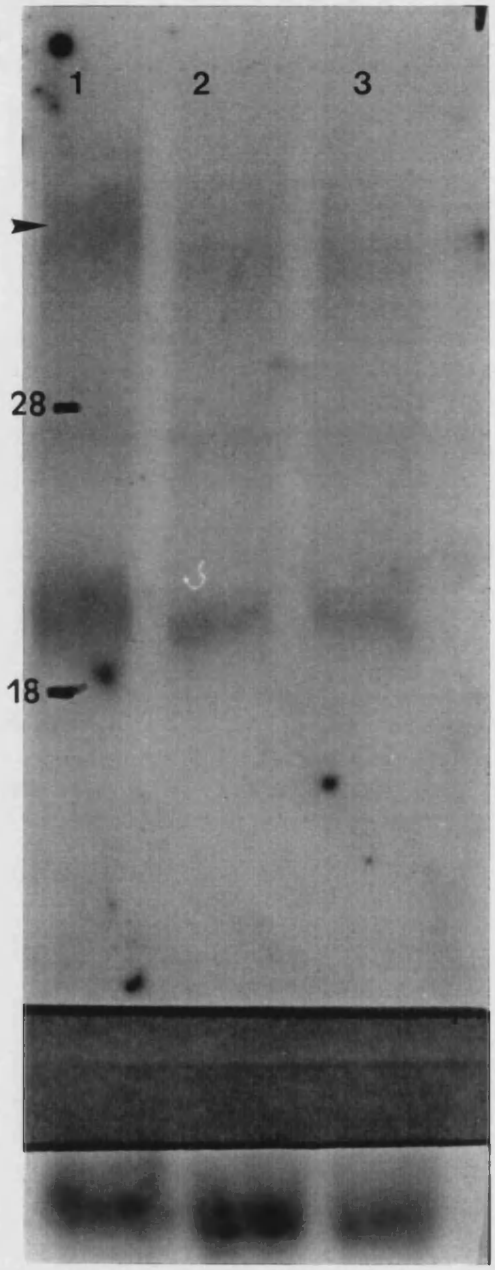


Figure III.5. The induction of TGFβ mRNA by 1,25-dihydroxyvitamin D_3.

Northern blot analysis was carried out on total RNA prepared as described in Materials and Methods. Ten micrograms of total RNA was loaded on each lane.

Lane 1 shows the treatment with 10^{-8}M 1,25-dihydroxyvitamin D_3 for 8 hours. Lane 2 shows the treatment with 10^{-8}M retinoic acid for 8 hours. Lane 3 shows untreated cells. Note the presence of a TGFβ_3 transcript (arrow). All lanes were normalized using a β-actin cDNA probe as shown in the panel below.

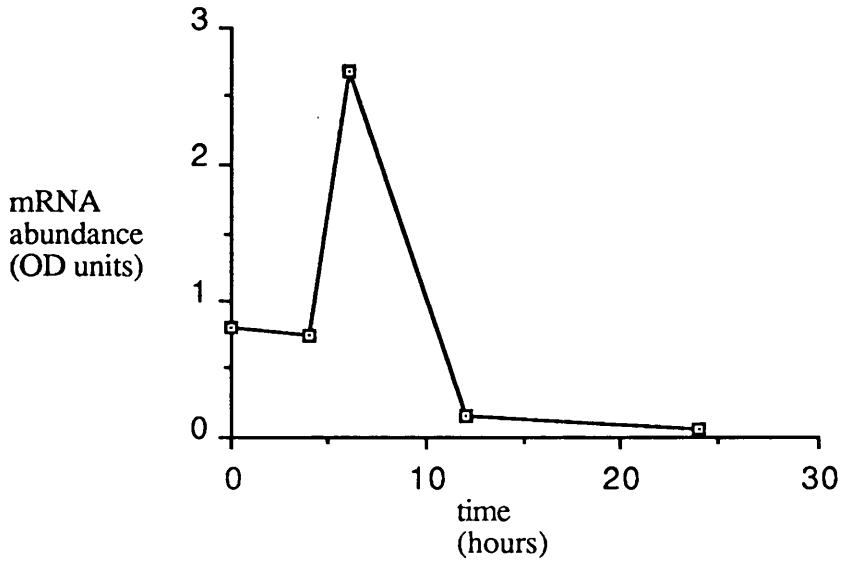


Figure III.6. The time course for 1,25-dihydroxyvitamin D_3 induction of TGFβ mRNA. Total RNA was prepared from cells treated with 10^{-8}M 1,25 D_3 for 4, 8 , 12 and 24 hours. The RNA was then loaded onto a dot blot and hybridized with a $TGF\beta_1$ probe. The mRNA was then quantified with a scanning densitometer. Each point represents a densitometry reading corrected for loading differences with a β-actin cDNA probe.

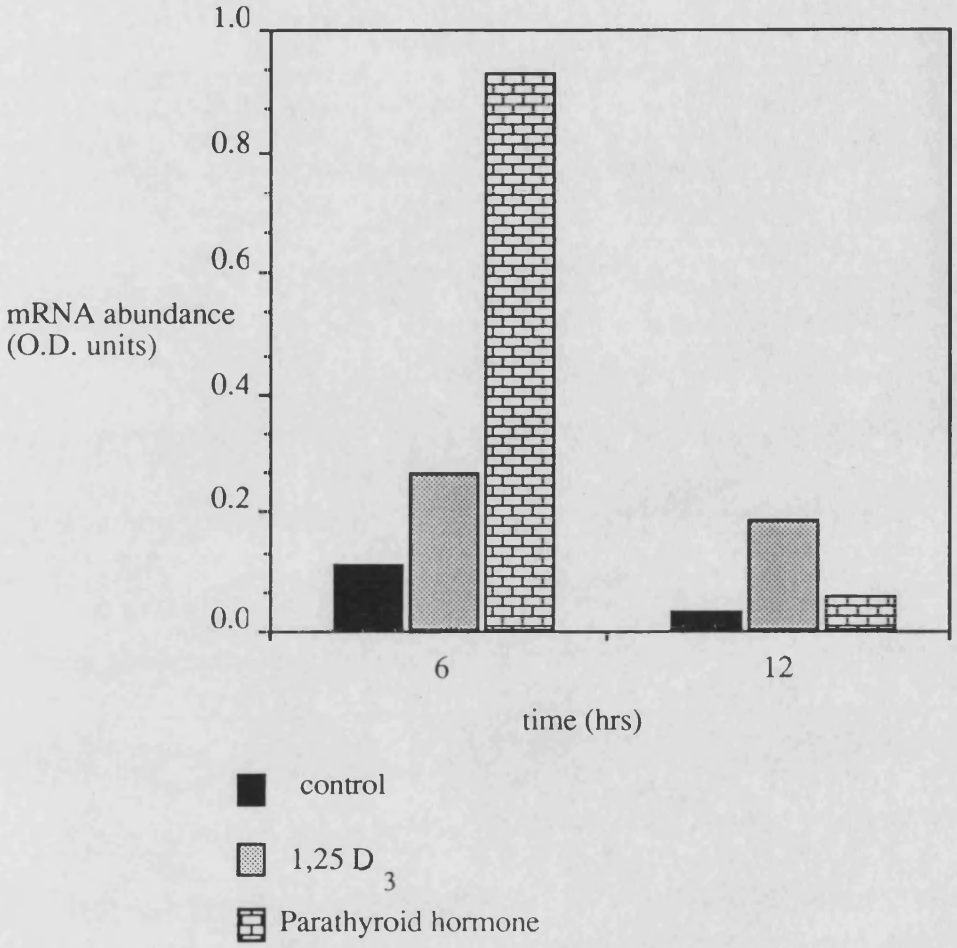


Figure III.7. Induction of TGFβ mRNA with 1,25 D_3 and parathyroid hormone. Messenger RNA was quantified in the same manner as for figure III.6. The data shows a direct comparison between treatment with 1,25 D_3 (10^{-8}M) and parathyroid hormone (10^{-7}M) for 6 and 12 hours on the same donor's cells .

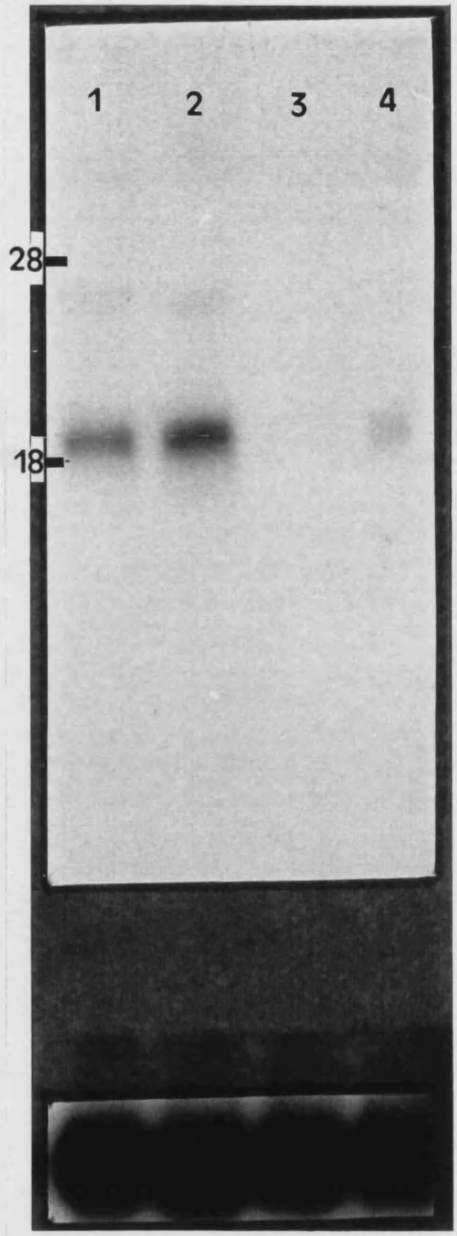


Figure III.8. The effect of hydrocortisone on TGFβ_1 mRNA expression.

Northern blot analysis was carried out on total RNA prepared as described in Materials and Methods. Ten micrograms of total RNA was loaded on each lane.

Lane 1, treatment with both 10^{-8}M hydrocortisone and 1,25 D_3 for 6 hours. Lane 2, treatment with 10^{-8}M 1,25 D_3 for 6 hours. Lane 3, treatment with 10^{-8}M hydrocortisone for 6 hours. Lane 4, no treatment. All lanes were normalized using a β-actin cDNA probe, as shown in the panel below.

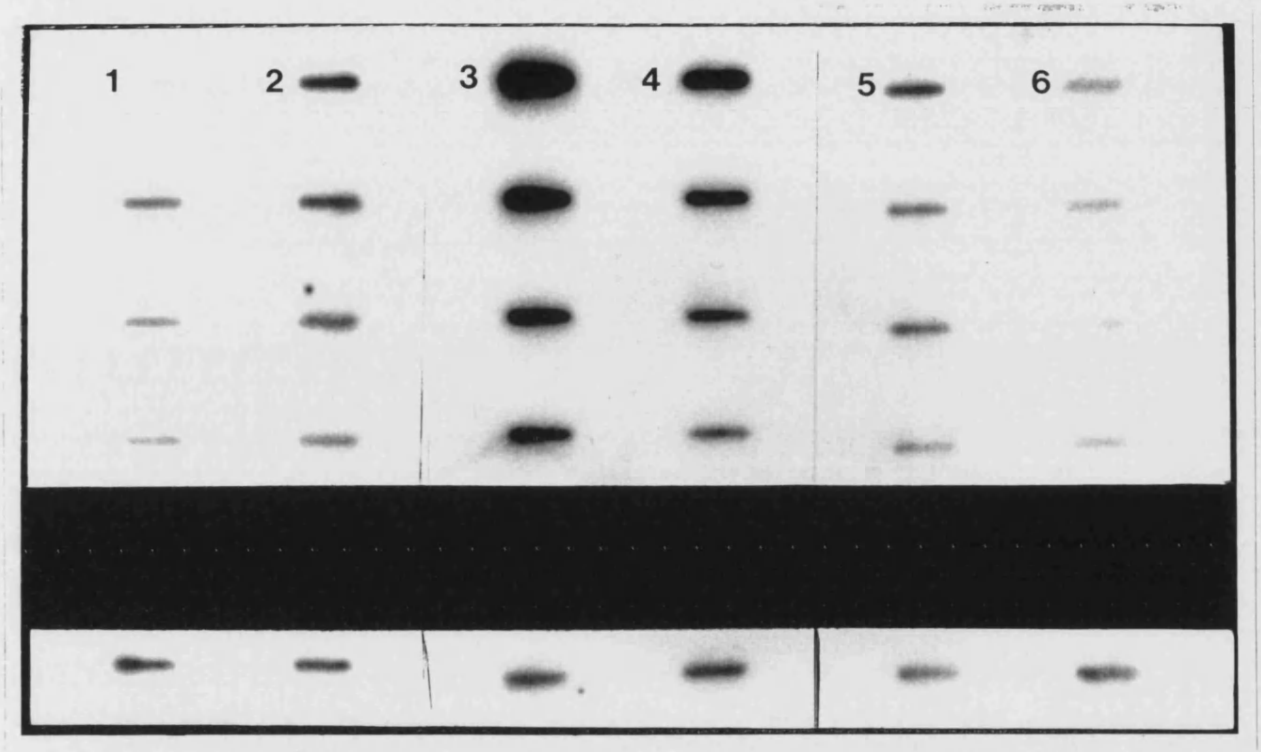


Figure III.9. The induction of TGFβ mRNA by 17-β oestradiol.

Slot blot analysis was carried out on total RNA prepared as described in Materials and Methods. The top slot contains 5 ug of total RNA, vertical slots below represent two fold serial dilutions of the samples.

Lanes 1 and 6: no treatment at 6 and 24 hours respectively.

Treatment with 17-β oestradiol (10^{-9} M) for 6, 8, 12, and 24 hours (lanes 2, 3, 4 and 5 respectively). All lanes were normalized using a β-actin cDNA probe, as shown in the panel below.

The effect of 17-β oestradiol on TGFβ expression in human osteoblasts

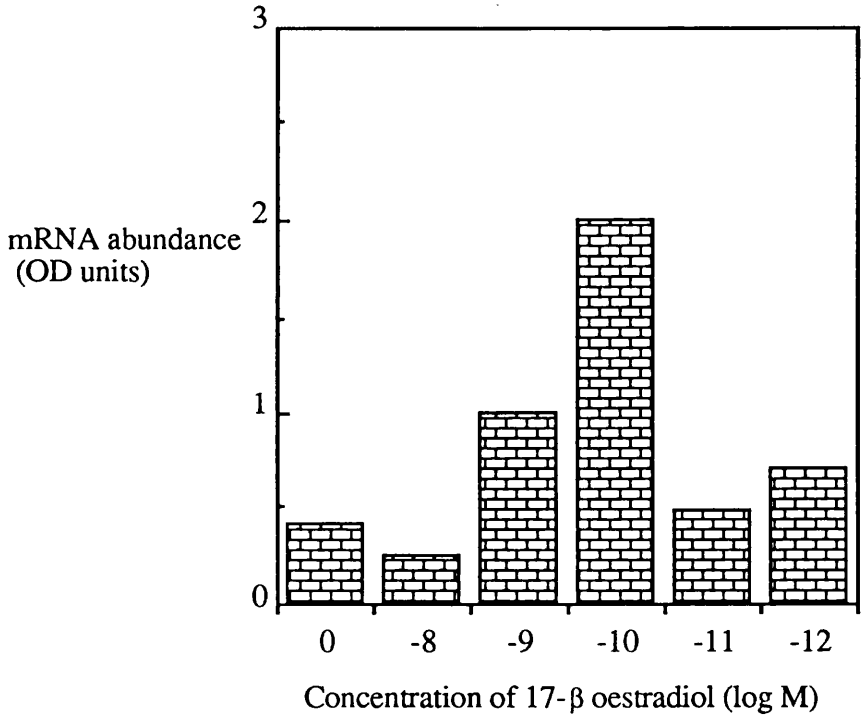


Figure III.10 Effect of 17-β oestradiol dose on induction of TGFβ mRNA

Total RNA was prepared from cells treated with 10^{-8}M, 10^{-9}M, 10^{-10}M, 10^{-11}M and 10^{-12}M 17-β oestradiol for 9 hours. The RNA was then loaded onto a Northern blot and hybridized with a TGFβ probe. The mRNA hybridization signal was then quantified with a scanning densitometer. Each point represents a densitometry reading corrected for loading differences with a β-actin cDNA probe.

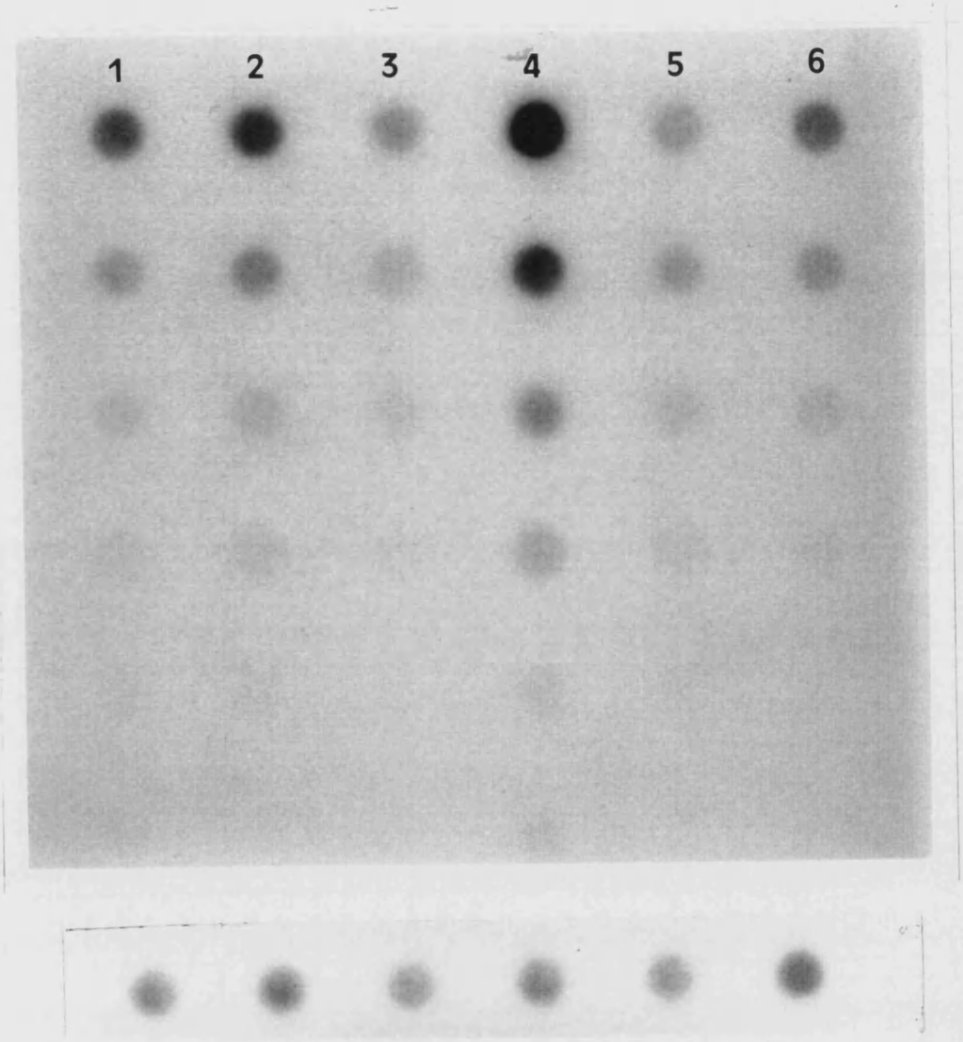
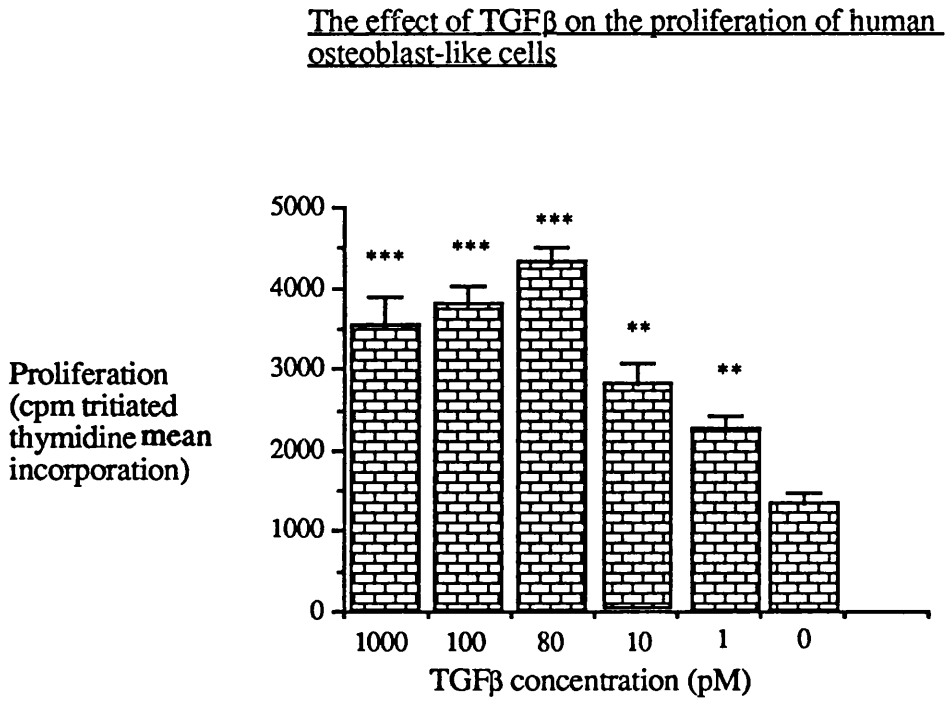


Figure III.11. Autocrine induction of TGFβ mRNA in human osteoblast-like cells.

Total RNA was extracted from cells as described in Materials and Methods, 5 ug were loaded into the top dot of the manifold, subsquent vertical dots represent two fold dilutions of the RNA sample.

Lanes 1 and 6: untreated cells at 6 and 12 hours, lanes 2 and 3 treatment with 10pM TGFβ for 6 and 12 hours respectively. Lanes 4 and 5 treatment with 100pM TGFβ for 6 and 12 hours respectively.



n = 4

Figure III.12. The effect of TGFβ on the proliferation of osteoblast-like cells in culture. The proliferation assay was carried out as described in chapter 2. Purified TGFβ was added in doses ranging from 1 pM to 1000 pM to triplicate wells of cells for 24 hours, after which the cells were "pulsed" with 1uCi of tritiated thymidine for 6 hours. After the pulse period the cells were lysed and the radioactive DNA harvested and quantified by liquid scintillation counting. ** and *** denote degree of significant differences between control and treatments as judged by the Student's T-test . ** = $p< 0.01$ and *** = $p<0.001$. Vertical bars represent standard errors.

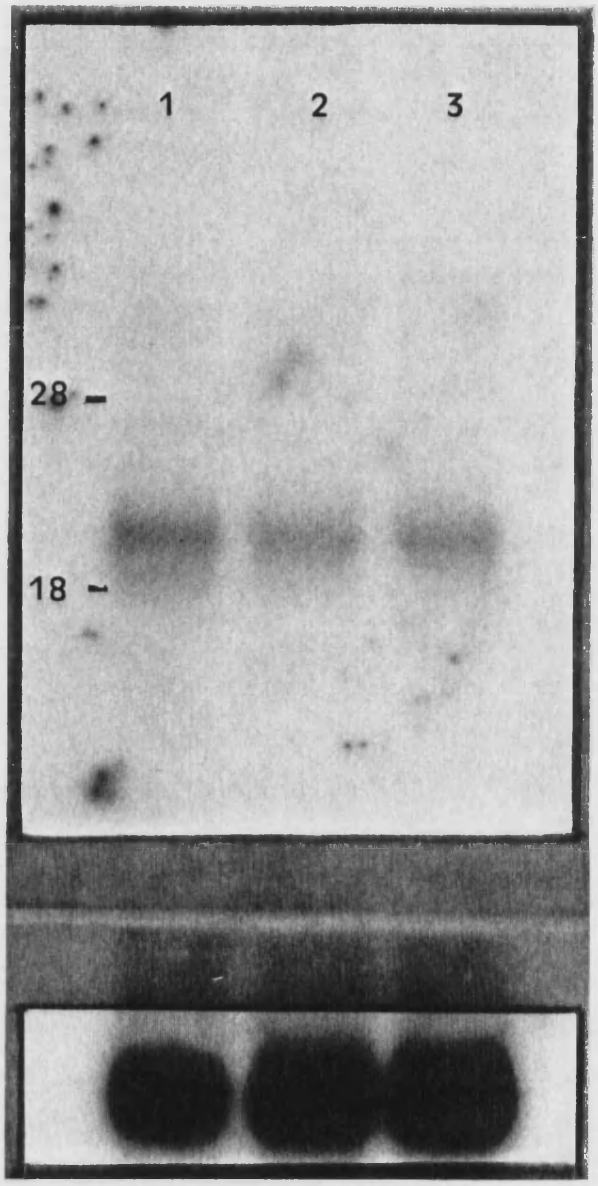


Figure III.13. The effect of IL-1α and LPS on TGFβ mRNA expression.
Northern blot analysis was carried out on total RNA prepared as described in Materials and Methods. Ten micrograms of total RNA was loaded on each lane.
Lane 1, no treatment. Lane 2, 200pg/ml IL-1α for 6 hours. Lane 3, 500ng/ml LPS for 6 hours. All lanes were normalized using a β-actin cDNA probe, as shown in the panel below.

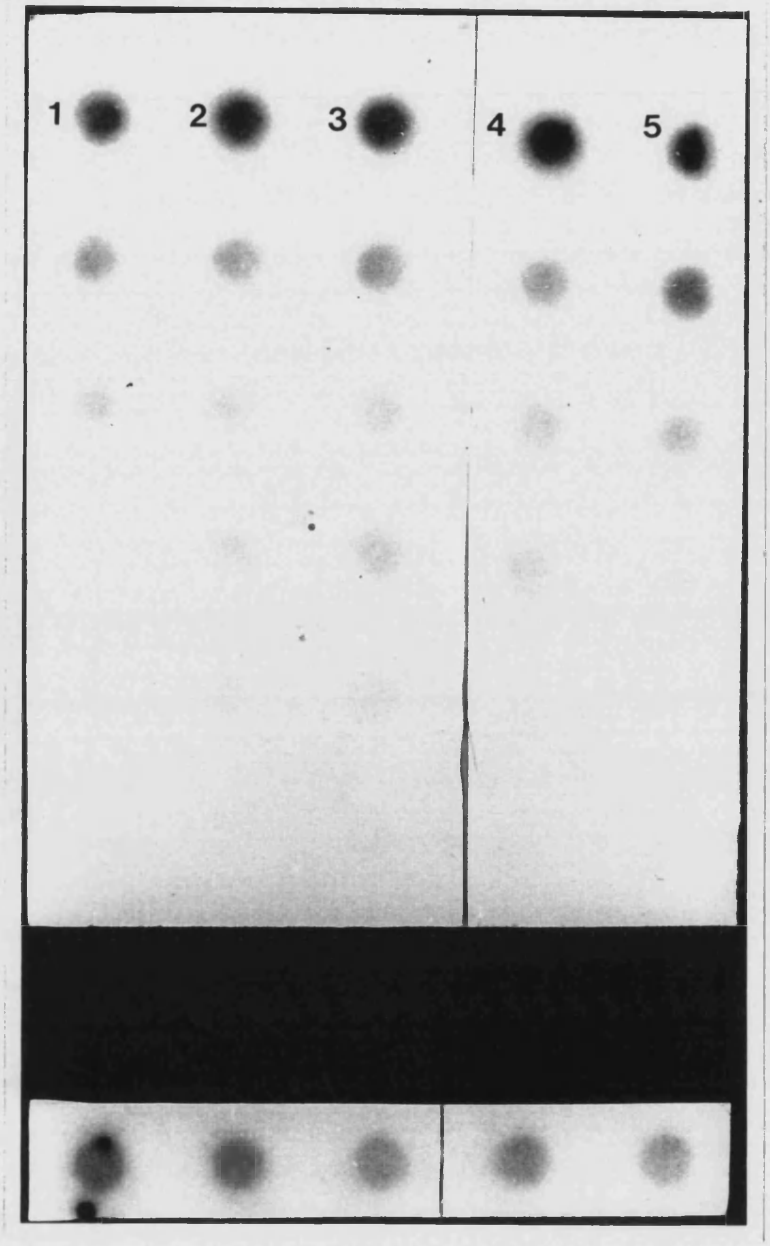


Figure III.14. The effect of TNFα on TGFβ mRNA expression.

Total RNA was extracted from cells as described in Materials and Methods. Five micrograms of RNA were loaded into the top dot of the manifold, subsequent vertical dots represent two fold dilutions of the RNA sample.

Lane 1 and 5 untreated cells at 6 and 24 hours, lanes 2, 3, and 4 treatment with 17ng/ml TNFα for 6, 12, and 24 hours respectively. All lanes were normalized using a β-actin cDNA probe, as shown in the panel below.

SUMMARY

TGFβ mRNA expression by human osteoblast-like cells

Treatment	Expression
(i) Systemic growth factors	
1,25 D_3	⬆
PTH	⬆
17β -oestradiol	⬆
Retinoic acid	➡
Hydrocortisone	⬇
(ii) Cytokines	
IL-1α	➡
TNFα	➡
TGFβ	⬆
LPS	➡

Key

⬆ = increase in mRNA expression

➡ = no effect on mRNA expression

⬇ = decrease in mRNA expression

III.6 DISCUSSION

The data presented, obtained using a variety of recombinant DNA techniques, demonstrates that mature human osteoblast-like cells are capable of expressing $TGF\beta_1$ mRNA. It is interesting to note that human osteoblast-like cells constitutively expressed TGFβ mRNA, as opposed to transient expression which is typical of many cytokines. This may be due to the fact that TGFβ is secreted as an inactive propeptide.

TGFβ mRNA was detected in all the osteoblast-like cells in the population, in contrast with the finding that only 20% of the cells expressed TGFβ protein. This observation suggests there may be some post-transcriptional control mechanism. Such a mechanism could be important in the microenvironment of remodelling. For example, it may be advantageous that only certain populations of osteoblasts synthesize TGFβ protein at any one time. Using this mechanism TGFβ deposition in the bone matrix by osteoblasts could be controlled in a highly localized manner.

Various cytokines and osteotropic factors were investigated to determine whether they modulated the expression of TGFβ mRNA. Generally the factors fell into two groups: TGFβ mRNA levels were induced by the systemic hormones 1,25 D_3, 17-β oestradiol and parathyroid hormone, at physiological concentrations, but remained unaffected by the cytokines IL-1α andTNFα at concentrations which previously had been shown to stimulate proliferation and prostaglandin release in these cells. Retinoic acid, an agent implicated in the maturation of osteoblasts, failed to induce TGFβ mRNA in physiological conditions in contrast to 1,25 D_3 which is also a differentiating agent.

The increase in TGFβ mRNA following 1,25 D_3 treatment was not very great when compared with the large increase obtained by PTH stimulation. This may suggest that the increase is due to a secondary phenomenon, and may not be directly biologically relevant. The slight differences in time courses for the 1,25 D_3 induction of TGFβ mRNA may be due to the fact that the bone cells derived from different donors contain populations of cells at different stages of differentiation. Alternatively, since some of

the bone was obtained from very old donors, it is possible that some populations of osteoblast-like cells could have reached a more senescent stage of their life cycle, making them slow to respond to modulatory agents.

Kim et al. (1990) reported that TGFβ expression is autoregulated in the human adenocarcinoma cell line A-549. The data from this study also suggest that TGFβ may have an autocrine role in human bone cells. This would be a very good mechanism for amplifying a "bone formation signal" for human osteoblasts. The increase in transcription of TGFβ mRNA would not only result in more synthesis of TGFβ protein to create a cascade in TGFβ expression, but the increased TGFβ would also promote osteoblast proliferation and matrix synthesis. It is important to realise that the cascade can only occur if the TGFβ is activated in the osteoblast microenvironment or if it is synthesized in an active form. Western blotting of human osteoblast-like cell culture supernatants would determine whether these cells are capable of secreting active TGFβ.

The mitogenic nature of TGFβ on connective tissue cells is postulated to be due to a PDGF autocrine loop (Battegay et al.1990). At low doses TGFβ increases PDGF-AA release and expression of PDGF receptor subunits. These two effects result in a net increase in connective tissue cell proliferation. At high concentrations PDGF-AA is further increased, but a net inhibition of proliferation is observed because PDGF receptor is down-regulated by high PDGF-AA concentrations. The possibility of two autocrine loops being superimposed in human osteoblast-like cells gives rise to a massive potential increase in any signal that promotes TGFβ expression.

In agreement with studies in other cell types (Waage and Nakke, 1991), hydrocortisone markedly decreased the expression of $TGF\beta_1$ mRNA in human osteoblast-like cells. Interestingly, the addition of 1,25 D_3 to hydrocortisone treated cells results in a partial reduction of the inhibitory effect. This may suggest that 1,25 D_3 is competing with the hydrocortisone for some DNA regulatory element of TGFβ transcription.

It is interesting to speculate by which mechanisms the agents modulate the expression of TGFβ by osteoblast-like cells. It seems likely for the steroid like molecules that transcriptional control occurs at the DNA level in the nucleus. Using the criteria outlined by Evans et al. (1988), a steroid binding consensus sequence has been identified in the regulatory region of the $TGF\beta_1$ gene (Metcalf, personal communication). It is also possible that these agents may act by firstly inducing the synthesis of a trans-acting factor which then increases TGFβ mRNA expression. It has been shown for example that treatment with TGFβ stabilizes type I procollagen mRNA by inducing the synthesis of a trans-acting factor (Raghow et al. 1987).

The finding that TGFβ expression in human osteoblast-like cells is increased by 17-β oestradiol treatment may have very important consequences in understanding post menopausal osteoporosis. The thinning of bone mass associated with this disorder is thought to be due to decreases in circulating oestrogen levels. It is possible that decreased oestrogen levels might reduce the synthesis of TGFβ by human osteoblasts. Less TGFβ would then become incorporated into the bone matrix and might lead to impaired coupling in subsequent remodelling cycles.

The data presented supports the hypothesis that the systemic hormones may be important regulatory agents for cytokine synthesis in bone. This study demonstrates that all the systemic hormones studied: 1,25 D_3, PTH, hydrocortisone and 17-β oestradiol modulate TGFβ mRNA expression. This suggests that the endocrine status of an individual may regulate TGFβ expression by human osteoblasts and act as another regulatory mechanism in the bone remodelling cycle. Since systemic hormones are not locally produced and their levels do not fluctuate quickly, it is likely that they have a role in bone formation rather than resorption.

It is conceivable that there are different levels of control of bone cell activity. Thus, a TGFβ–like factor would be produced and incorporated into the bone matrix throughout the skeleton over a prolonged period under the influence of the endocrine system of the individual. It may then be activated at a much later time in a highly localized fashion

(such as under an osteoclast). Indeed, Finkelman et al. (1991) have shown that vitamin D deficiency causes a selective reduction in the amount of TGFβ in rat bone matrix. A similar selective depletion in TGFβ content in the bone matrix has also been demonstrated in ovariectomized mice.

Finally, it is important to realise that these studies look at the modulation of one factor after treatment with another factor, this may only highlight a small fraction of all the interactions taking place at any one time. It is therefore incorrect to equate mRNA levels directly with bioactivities, as other inhibitors and trans acting factors may also be present.

Chapter IV : The expression of IL-1 by human osteoblast-like cells *in vitro*.

IV.1 ABSTRACT

IL-1 was the first immune cytokine to be positively identified as having an important action in the control of bone turnover. IL-1 expression by osteoblasts from several species has been documented. This study was undertaken to characterise human osteoblastic IL-1 expression and to investigate the mechanisms by which this important cytokine is modulated.

The expression of mRNA for IL-1 was studied in human osteoblast-like cells *in vitro*. These cells did not constitutively express either IL-1α or IL-1β mRNA. The cytokines IL-1α (200pg/ml), TNFα (17ng/ml) and the bacterial lipopolysaccharide (LPS) (500ng/ml) stimulated the production of IL-1β mRNA, but not IL-1α mRNA. The maximal stimulation of IL-1β mRNA occurred after 6 - 8 hours, the mRNA then quickly fell to undetectable basal levels. This was followed by an increase in protein production after 24 hours. IL-1β mRNA expression was detected at the cellular level, using *in situ* hybridization techniques. Immunolocalization studies confirmed that a small proportion of the total population of human osteoblast-like cells were expressing IL-1 protein.

Treatment of the cells with the systemic hormones 1,25 D_3 (10^{-8}M), 17-β oestradiol (10^{-7} -10^{-12}M) and parathyroid hormone (PTH) (10^{-7}M) did not affect the production of IL-1β mRNA. Retinoic acid (10^{-8}M), an osteoblast differentiation agent, also had no effect on IL-1β mRNA expression. The addition of hydrocortisone (10^{-8}M), resulted in the complete suppression of IL-1α-induced IL-1β mRNA expression.

The human osteoblast-like cells were also screened to determine whether they expressed IL-1 antagonist protein (IRAP) mRNA. No IRAP mRNA was detected in untreated cells, or in cells stimulated with the cytokines; IL-1α, TNFα, TGFβ, or LPS. The systemic hormones; 1,25 D_3, 17-β oestradiol, hydrocortisone and PTH also failed to affect the expression of IRAP mRNA.

IV.2 INTRODUCTION

Interleukin 1 (IL-1) exists in two forms termed IL-1α and IL-1β, which are structurally related and bind to the same receptor (March et al. 1985). Although the two proteins share only small stretches of amino acids and are not highly homologous (26%), they have similar actions on a wide range of cellular targets. The genes for both forms are located on chromosome 2 and consist of seven exons (Clark et al.1986; Furutani et al.1986). The coding sequence encodes a 31 kDa precursor form which must be enzymatically cleaved to give the active 17 kDa species.

A third member of the IL-1 gene family has been recently identified, which shows IL-1 antagonist activity, namely IRAP (IL-1 receptor antagonist protein) (Eisenberg et al.1990; Mazzei et al. 1990). This 26 kDa protein was initially purified from culture supernatants of IgG stimulated monocytes, and has been shown to block IL-1 activity by binding to the IL-1 receptor (Arend et al.1989). In common with the other members in the family IRAP is only slightly structurally related, it is 26% homologous with IL-1β, and 19% with IL-1α (Arend et al.1989). Other non characterised high molecular weight IL-1 inhibitors exist, including a 98kDa protein released by human gingival epithelial cells (Walsh et al.1987), and a 40kDa factor produced by u.v irradiated murine epidermal cells (Schwarz et al.1987).

IL-1 is synthesized by a wide variety of cells. These include; those of connective tissue origin such as synovial fibroblasts, keratinocytes, fibroblasts and chondrocytes; immune cells including B cells, T cells, NK cells, monocytes, macrophages and neutrophils; and cells of neuronal origin, astrocytes, microglia and glioma cells (for review see Dinarello, 1988). The stimulus required for IL-1 synthesis varies according to the cell type, but includes cytokines (Littlewood et al.1991a), infectious agents such as bacteria and viruses (Giovine and Duff, 1990), and even inorganic substances such as bone fragments (Pacifici et al.1990).

Evidence has recently been presented which suggests that different activation signals are required for the expression of IL-1α and IL-1β genes (Hurme and Serkkola, 1991).

A study with human peripheral monocytes showed that in contrast with LPS, which induced the production of high levels of both IL-1α and IL-1β, the protein kinase C activator phorbol myristic acid (PMA) induced only IL-1β synthesis. Nuclear "run off" assays revealed that protein kinase C activation alone was sufficient for the induction of the IL-1β gene, but not the activation of IL-1α transcription. The proportion of IL-1α to IL-1β produced is dependent on the cell type and in the majority of cases IL-1β seems to be synthesized in a great excess to IL-1α (Auron et al.1984). It has been suggested that IL-1α is not secreted like IL-1β, but may remain associated with the cell membrane (Auron et al.1984). The concept of a membrane bound form of IL-1 is thought to explain the ability of IL-1 to participate in localized paracrine and autocrine events (for example antigen presentation), without inducing systemic effects.

IL-1 was one of the earliest cytokines to be identified as having a regulatory role in bone cell function, and remains the most potent bone resorbing agent so far described (see chapter I for review). It was postulated that the resorption was mediated by an increase in osteoclast numbers, rather than an increase in the activity of existing osteoclasts, since there was a 24 hr "lag phase" between IL-1 stimulation and enhanced resorption (Gowen et al.1986). This idea prompted a study to determine the effect of IL-1 on osteoclast formation in long term marrow cultures. Both IL-1α and IL-1β were shown to stimulate the formation of multinucleated cells in a similar manner to CSFs, ie. they were only required to be present during the initial week of the 3 week culture period (Pfeilschifter et al.1989). It is also possible that IL-1 stimulated resorption is mediated via other cell types. Studies by Thomson et al (1986) showed that isolated osteoclasts were only capable of resorbing devitalized bone in the presence of cells of the osteoblast lineage. They postulated that IL-1 induces the synthesis of an osteoclast activating factor (OAF) by the osteoblasts, although no such factor has ever been unequivocally identified.

Anabolic effects of IL-1 in bone metabolism have also been documented. IL-1 has been shown to stimulate proliferation in primary cultures of human osteoblasts (Evans

et al.1990). This finding was supported by Rickard et al. (1991) who used a method which involved tritiated thymidine labelling to reveal proliferation, in conjunction with the osteoblastic marker alkaline phosphatase. This method revealed that IL-1 stimulated the proliferation of a specific preosteoblast population. In contrast, the proliferation of other osteoblastic cell lines including normal mouse 3T3-E1 cells and the osteosarcoma line MG63, is reported to be inhibited by IL-1 treatment (Hanazawa et al.1986; Dedhar, 1989). IL-1 has also been shown to be inhibitory in an *in vitro* model of bone formation. Long term IL-1 treatment of cultures of fetal rat osteoblastic cells, resulted in a decrease in the number of spontaneously formed mineralised nodules (Stashenko et al.1987).

The effects of IL-1 on the synthesis of bone proteins seems to be bidirectional. IL-1 has been shown to increase collagen synthesis in organ cultured bones (Canalis et al.1986), but decrease 1,25 D_3 stimulated production of osteocalcin in human osteoblast-like cells (Evans et al.1989). In addition, both basal and 1,25 D_3 induced alkaline phosphatase expression by human osteoblast-like cells is reduced by IL-1 treatment at low doses (Evans et al.1989). Overall IL-1 seems to suppress the expression of a differentiated osteoblastic phenotype whilst promoting the proliferation of osteoblast precursors. Possibly this might be a mechanism whereby bone formation is coupled to IL-1 induced resorption.

IL-1 has also been implicated in a number of bone diseases such as osteoporosis and myeloma. Peripheral monocytes from patients with a high rate of bone turnover associated with osteoporosis, have been shown to release more IL-1 than those patients with low bone turnover (Pacifici et al.1987). Additionally Pioli et al. (1989) demonstrated that mononuclear cells from patients with Pagets disease when stimulated with LPS *in vitro* released more IL-1 than age matched controls. However the relevance of systemic levels of IL-1 to diseased states is questionable, therefore it was important to note that blood cells removed from the illiac crest of these patients also spontaneously released IL-1. Finally, IL-1 has been implicated with the local bone

destruction that is often associated with myeloma. Myeloma cells from some patients with local bone damage have been shown to release large amounts of IL-1β (Kawano et al.1988).

Preliminary reports have shown that human osteoblast-like cells are capable of producing a factor with IL-1-like activity (Hughes et al.1988; Keeting et al.1989), although this factor has not been identified. Studies on cytokine release from osteoblasts of various species show that cytokine expression is often regulated in a species or cell line specific fashion. For example it has been shown that IL-6 expression is stimulated by PTH in rat, but not human osteoblast-like cells (Feyen et al.1989; Littlewood et al.1991b). For this reason human osteoblast-like cells were studied to verify the production of IL-1 by human osteoblasts, and to determine how the expression of IL-1 mRNA is modulated by a range of osteotropic hormones and cytokines. Human osteoblast-like cells derived by outgrowth from trabecular bone obtained at surgery were cultured *in vitro*. These cells have been widely used for many years as a model of human osteoblasts, and most recently to study cytokine production (Gowen et al.1990). Using this approach it was hoped to gain a greater insight into the complex signalling between cell types during human bone remodelling.

IV.3 METHODS AND MATERIALS

Northern and slot/dot blots

Human osteoblast-like cells were obtained from explants of bone (as described in chapter II.1(i)), and were prepared as described in chapter III.2. For the Northern and slot blot experiments 10 -12 petri dishes of osteoblast-like cells were used for each experiment. All treatments were undertaken in a synthetic serum-free medium (1xMEM, 0.1% BSA, 10ug/ml transferrin, 1ug/ml insulin, $3x10^{-8}$M sodium selenate, 1mM glutamine, 100U/ml penicillin /streptomycin), since serum contains components such as α_2 macroglobulin which have been shown to bind a spectrum of different cytokines. After treatment, the medium was removed from the petri dishes and stored

at -20°C for the IL-1 bioassay (see chapter II.1.(ii)). The RNA was harvested as described in chapter III and purified according to a method based on that of Chomininski et al. (1987) (see Chapter II 2(i)). Routinely 2 petri dishes were used per treatment, yields of approximately 30 - 50 ug RNA /1x10^{-6} cells were normally obtained.

The IRAP probe was prepared from a plasmid construct (pGEM-IRAP-P5) which contained a 1.8 cDNA fragment of the coding sequence. The cDNA insert was liberated by using the restriction enzyme EcoR1 (see Chapter 2 for restriction digests), and "gene cleaned" before random hexanucleotide radiolabelling.

The Northern blots and slot/dot blots were carried out as described in chapter II, 2(ii) and 2(iii) . Total cellular RNA (10 ug) was used per lane for Northern blots, and 5ug for the highest concentration in slot blots. All filters were stained with methylene blue (see chapter II 2(iv)) to check for equal loading. The hybridization was carried out according to a method based on that of Goldberg et al. (1979) (chapter II 4(ii)). After post hybridization washing, the filters were exposed to X-ray film for 1 - 3 days.

In situ hybridization

Human osteoblast-like cells were trypsinized and seeded at a density of 3000 /spot on multiwell slides for *in situ* hybridization. After 24 hours the cells were treated with IL-1α (200pg/ml) or incubated with media for a further 6 hours, then washed with PBS and fixed in 4% paraformaldehyde/PBS for 5 mins. The slides were then dehydrated through an ethanol series and stored for up to 1 month at -20°C prior to hybridization. The IL-1β RNA probe was generated from the linearized pgem2il1beta plasmid which contained a 300 bp section of the cDNA. Sense and antisense transcripts were prepared using SP6 and T7 RNA polymerase promoters (see chapter VI.3). The specific activities of the RNA probes were checked to prove they were comparable (chapter II 4(i)). The length of both probes were verified by vertical polyacrylamide gel electrophoresis (chapter II 2(vi)) to check that the majority of the transcripts were of full

length. The *in situ* hybridization of the IL-1β RNA probe was carried out (as described in chapter III 4(iii))

Immunolocalization

For immunolocalization studies the cells were stained using a murine IL-1β monoclonal antibody (as described in chapter II 1(iv)).

IL-1 bioassay

The IL-1 bioassay was carried out as described in Chapter II 1(ii). Cell supernatants were obtained from the Northern blot experiments ($1x10^6$/dish) and cultures of 48 well dishes, which had been seeded with bone cells from the same donor ($2x10^5$/well). Triplicate wells were assayed in duplicate for the 48 well plate cultures.

Preparation of peripheral blood polymorphonuclear cells (PBMCs) from human blood

Peripheral blood (30 ml) was obtained from normal healthy volunteers, and dispensed into heparinized (100u/ml) tubes. After diluting 1:1 with sterile PBS, the blood cells were separated by density-gradient centrifugation on Ficoll-hypaque. The mononuclear cell layer was extracted from the serum/Ficoll-hypaque interface. Cells were washed twice in media (1xRPMI, 10%FCS, 1mM glutamine, 100U/ml penicillin /streptomycin), and resuspended at a density of $1x10^6$/ml. For the IRAP studies cells were dispensed into T25 culture flasks and treated with PMA (1nM) or left untreated. Subsequently, an aliquot of PMA stimulated cells were stimulated with 500ng/ml LPS. Finally the PBMCs were harvested by centrifugation, and lysed in guanidine chloride solution for RNA extraction.

IRAP induction in U937 cells

U937 cells were dispensed at a density of $2x10^6$/ml into T25 culture flasks. The cells were treated with PMA for 48 hrs, which induced the cell suspension to differentiate

into adherent cells. After this period the cells were treated with 500ng/ml LPS for a further 6 hours. The adherent cells were then harvested into guanidine hydrochloride solution with a sterile scraper for RNA extraction.

IV.4 RESULTS

Northern blot analysis showed that there was no constitutive expression of IL-1β or IL-1α mRNA in human osteoblast-like cells. Messenger RNA for type I collagen (a bone matrix protein) was detected in large amounts (see figure IV.1), which confirmed that the cells were actively metabolising. No IL-1α mRNA expression was detected in the cells from more than 10 donors either with or without IL-1 stimulation (data not shown). IL-1β mRNA was detected when the human osteoblast-like cells were treated with IL-1α (200pg/ml) or the bacterial polysacharide LPS (500ng/ml) (see figure IV.2). Treatment with IL-1α gave a 20 fold increase in IL-1β mRNA after 6 hours. LPS was a less potent stimulating agent, giving rise to a 5 fold increase in IL-1β mRNA over the same time period.

A bioassay for IL-1 using the $D_{10}N_4M$ cell line was optimized. A typical standard curve obtained with rhIL-1β is shown in figure IV.3. This assay was used to quantify the amounts of IL-1 bioactivity produced by the LPS stimulated human osteoblast-like cells. There was a lag phase between IL-1 mRNA expression and the synthesis of IL-1 protein (compare fig IV.2 lane 2, and fig IV.4). IL-1 bioactivity was detectable at 12 hours and rose over a 48 hour time period. The amount of IL-1 bioactivity detected in the supernatants from LPS stimulated cells was very low (see figure 4).

The expression of IL-1β mRNA was confirmed at the cellular level by *in situ* hybridization. Figure IV.5a shows human osteoblast-like cells that have been stimulated IL-1α (200pg/ml) for 6 hours then hybridized with an IL-1β RNA probe. All the cells in the population were seen to express IL-1β mRNA. A sense IL-1β RNA probe was used as a negative control and gave very low levels of background hybridization (see figure IV.5b).

Immunolocalization studies were undertaken to confirm the expression of IL-1β at the protein level. The cells were treated with 0.5uM monensin which had previously been shown to increase IL-1 production (Rubartelli et al.1990). IL-1β expression was

detected in a very small proportion of the human osteoblastic cells (see figure IV.6a). The staining appeared to be perinuclear, and there was also some staining on the cell membrane (see figure IV.6b for high magnification photograph).

Another "inflammatory" cytokine TNFα (17 ng/ml) induced a transient increase in IL-1β mRNA. A peak in IL-1β mRNA expression was detected after 8 hours (figure IV.7), which fell sharply to basal levels after 12 hours. There was a lag phase between peak IL-1β mRNA expression and protein synthesis, since IL-1 bioactivity was not detected until 24 hours after stimulation (figure IV.8). Again the amount of IL-1 bioactivity that was detected was very low. In contrast, treatment with another cytokine TGFβ (10 - 100 pM) failed to induce IL-1β mRNA expression over a 24 hour time course (data not shown).

The systemic hormones had previously been shown to modulate TGFβ expression in human osteoblast-like cells (see chapter III). Therefore their effect on IL-1β mRNA expression was investigated. 1,25 D_3, PTH, and 17-β oestradiol treatments failed to stimulate IL-1β mRNA expression, which remained at a basal undetectable level (data not shown). Treatment with hydrocortisone, which had previously been shown to abrogate cytokine expression, resulted in the complete suppression of IL-1α-induced IL-1β mRNA expression (figure IV.9).

Since the stimulated-IL-1β mRNA levels appeared to be fairly abundant, but the bioactivity levels were low, populations of human osteoblast-like cells were screened for IRAP mRNA. It was thought that the IRAP might be interfering with the $D_{10}N_4M$ bioassay and giving artificially low IL-1 bioactivities. Conditions which induced IRAP expression in human peripheral blood mononuclear cells (PBMCs) and U937 cells, failed to stimulate IRAP mRNA expression in human osteoblast-like cells (see figure IV.10). No IRAP mRNA expression was detected in cells treated with the cytokines IL-1α, IL-1β, TNFα, TGFβ or the lipopolysacharide LPS (data not shown). Additionally the systemic hormones 1,25 D_3 (10^{-8}M), PTH (10^{-7}M), 17-β oestradiol (10^{-7} - 10^{-12}M) and hydrocortisone (10^{-8}M) failed to induce the expression of IRAP

mRNA (data not shown). Populations of human osteoblast-like cells from over 10 different donors were screened and none expressed IRAP mRNA.

IV.5 FIGURES

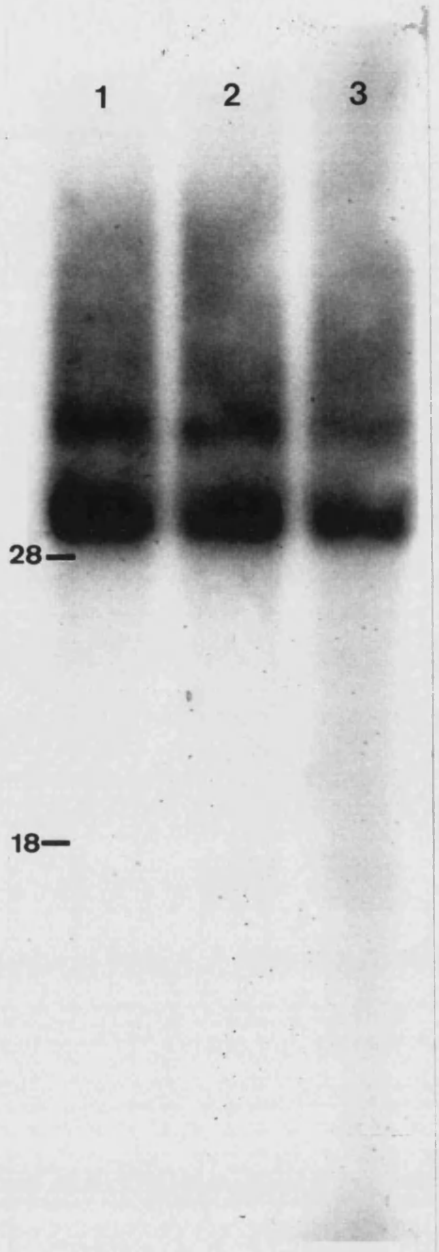


Figure IV.1: The expression of type I collagen mRNA by human osteoblast-like cells. Lanes 1, 2, and 3 represent RNA extracted from 3 different populations of bone cells. The filter was initially probed for IL-1β and IL-1α mRNA but none was detected. Subsequent hybridization with a type I collagen probe reveals high expression of two type I collagen mRNA species of approximately 4.9 and 5.2kb.

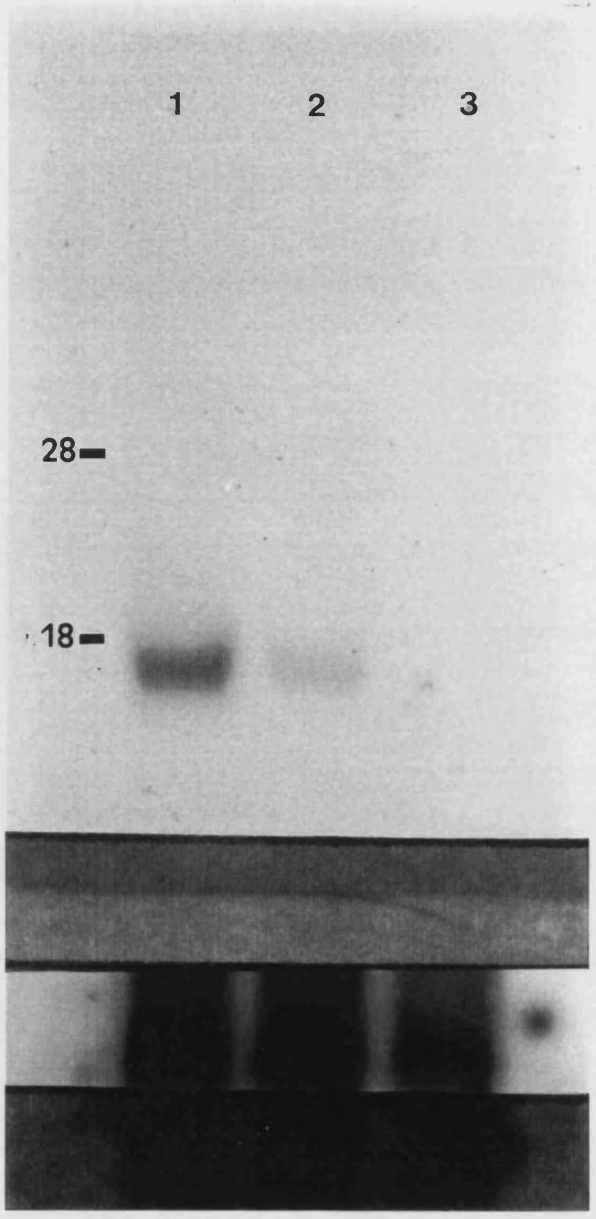


Figure IV.2. The induction of IL-1β mRNA by IL-1α and LPS

Northern blot analysis was carried out on total RNA prepared as desribed in Materials and Methods, ten micrograms of total RNA was loaded on each lane.

Lane 1 shows the treatment with IL-1α (200pg/ml) for 6 hours. Lane 2 shows the treatment with LPS (500ng/ml) for 6 hours. Lane 3 Untreated cells. A 1.8 kb transcript characteristic for IL-1β is present in lanes 1 and 2. All lanes were normalized using a β-actin cDNA probe, as shown in the panel below.

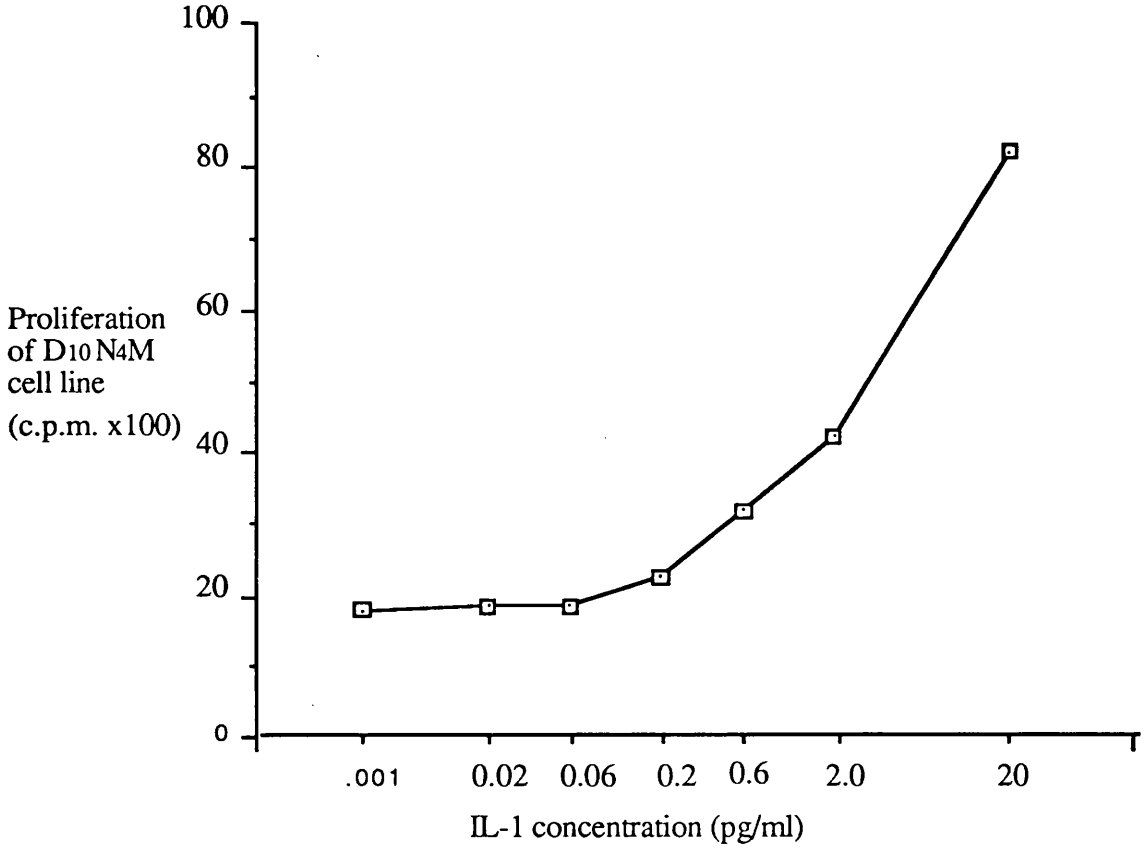
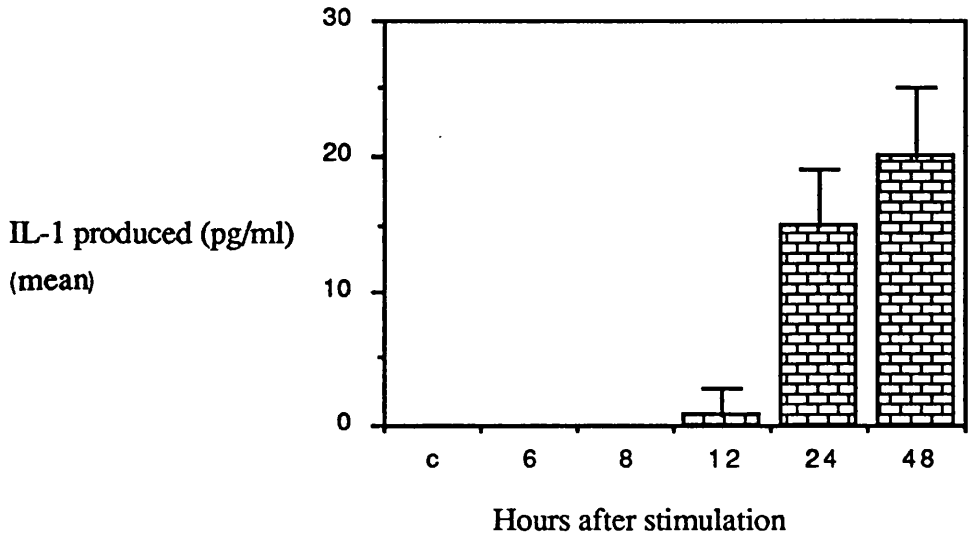


Figure IV.3. Standard curve for IL-1 bioassay

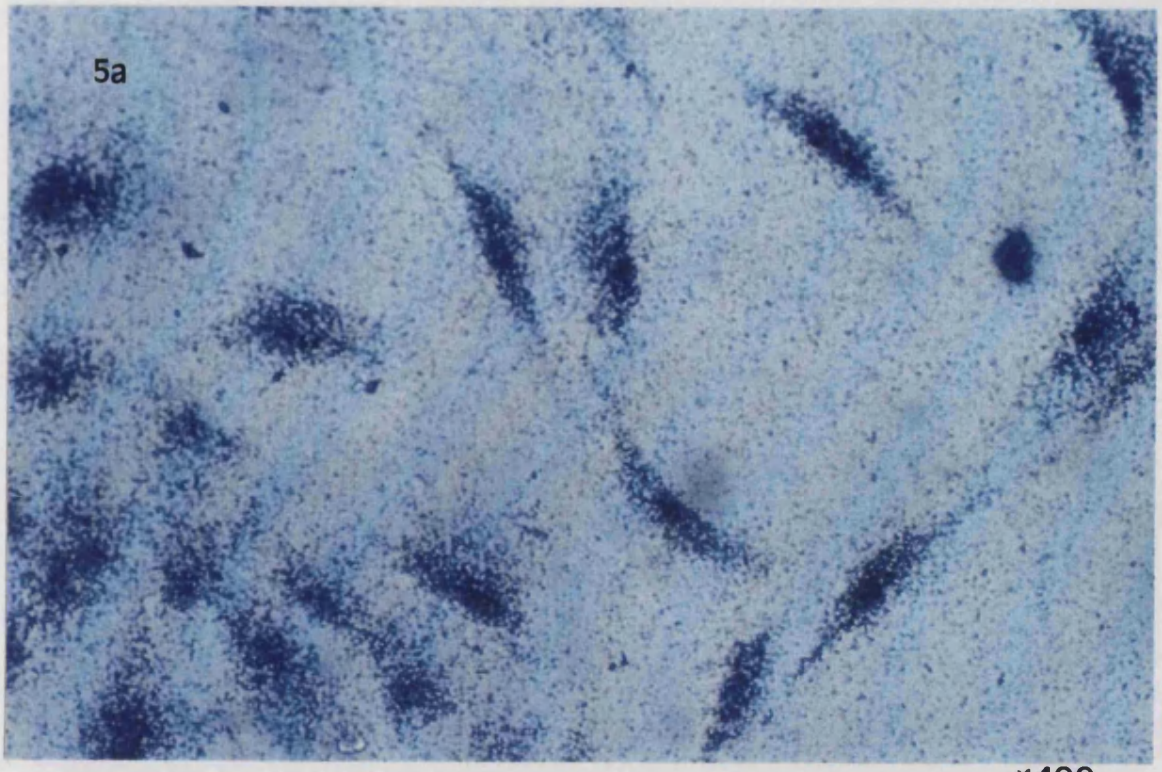
Human recombinant IL-1β was used to obtain a standard curve of IL-1 concentration versus $D_{10}N_4M$ cell proliferation. Each point represents 3 replicate wells, the standard curve can be seen to be useful over the range 0.2 - 20 pg/ml IL-1.

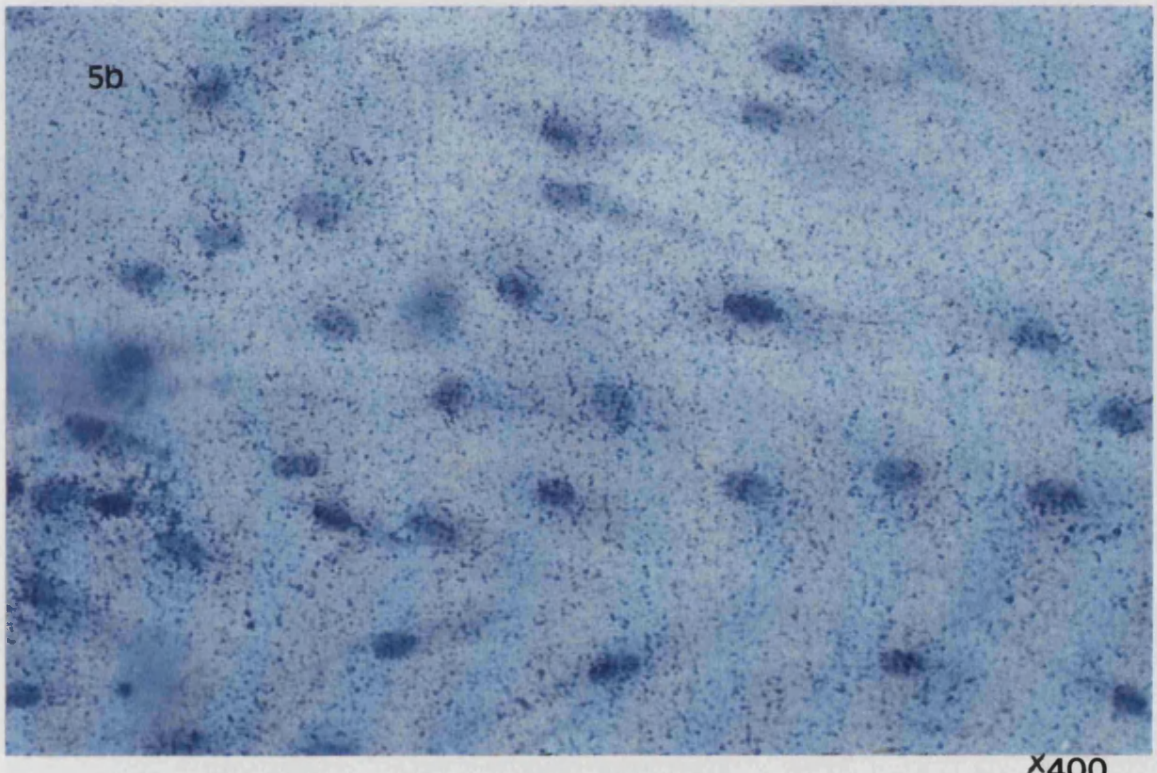
Figure IV.4: Induction of IL-1 bioactivity by LPS



n = 6

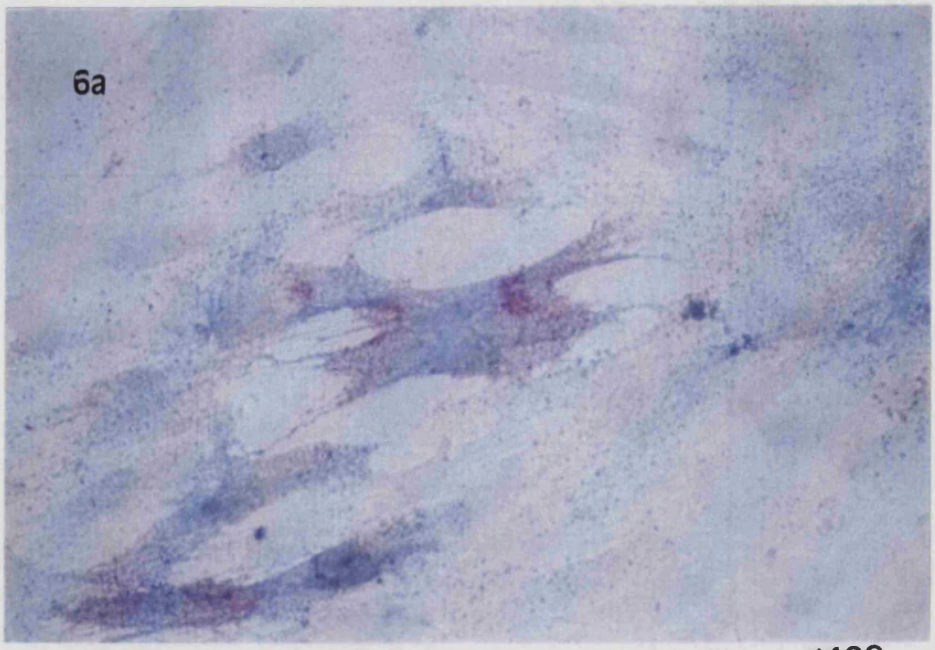
Cell supernatants were assayed for IL-1 bioactivity at 6, 8, 12, 24, and 48 hours after stimulation with 500ug/ml LPS. The results represent assays in duplicate of triplicate treatment supernatants. The letter c represents untreated cells after 48 hours in culture. The vertical bars represent standard errors.



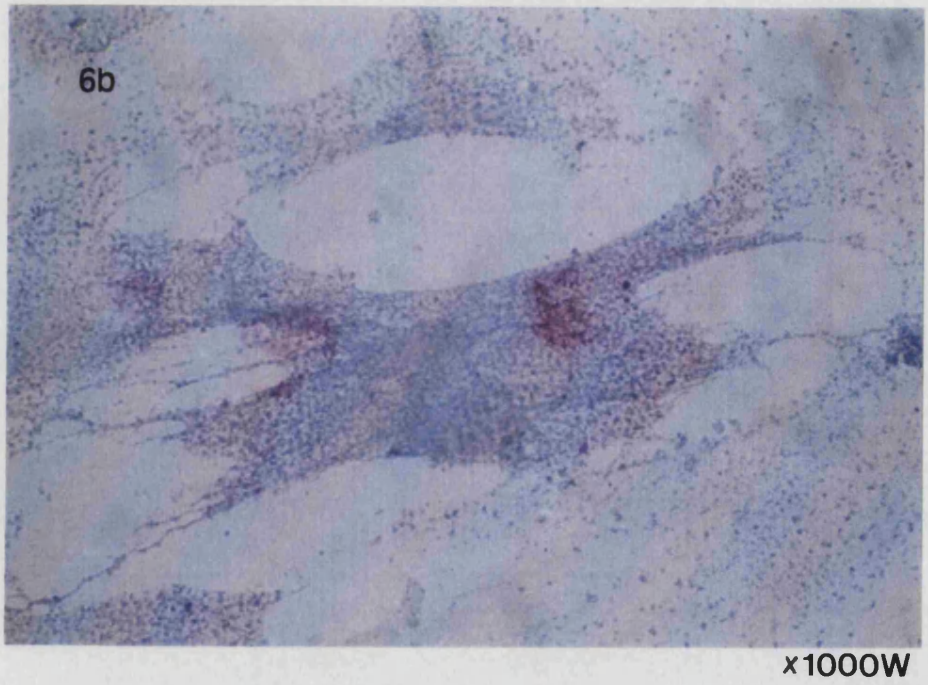


Figures IV.5a and IV.5b. *In situ* hybridization of IL-1β mRNA in human osteoblast-like cells.

The *in situ* hybridization was carried out as described in Materials and Methods. Grains represent binding of the antisense probe (5a). The entire population of osteoblasts can be seen to express IL-1β mRNA. Figure IV.5b shows that very little hybridization is observed with the sense negative control probe.



Figures IV.6a and IV.6b. Immunolocalization of IL-1β protein in human osteoblast-like cells in culture. Cells were cultured as described in Materials and Methods, and stained for endogenous alkaline phosphatase activity (blue stain). IL-1β expression (pink stain) was detected by using a mouse monoclonal antibody with an alkaline phosphatase conjugated secondary antibody. Figure IV.6b is a higher magnification photograph of a single cell and shows the perinuclear and cell membrane staining.



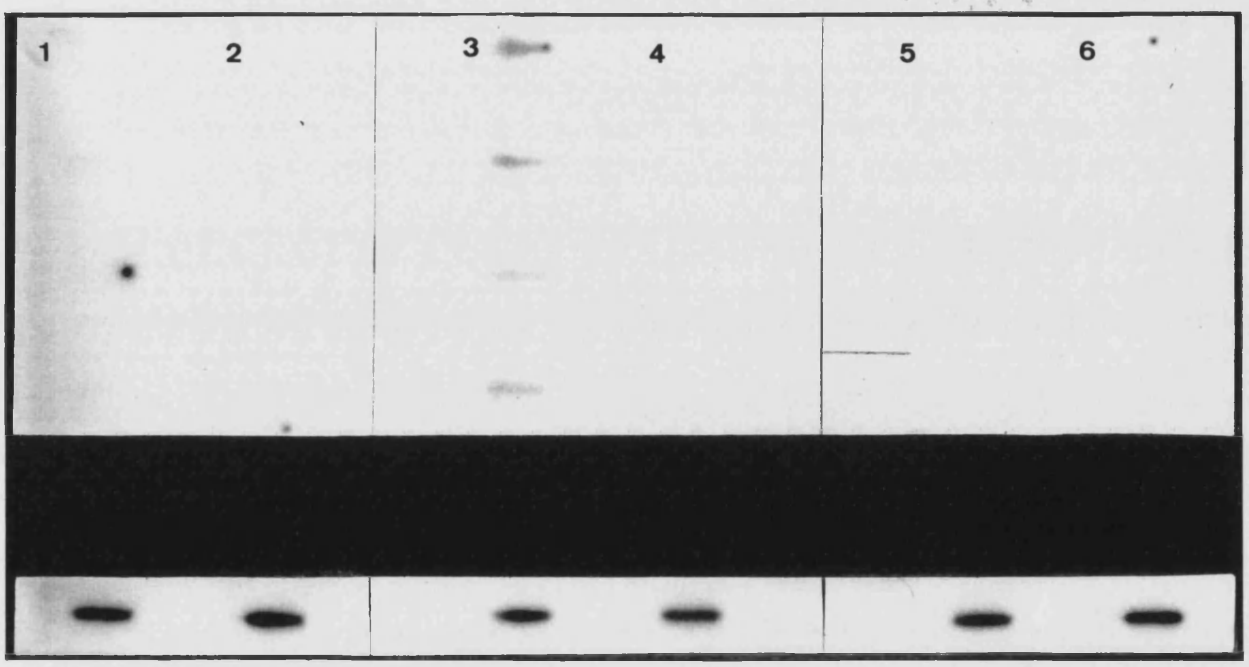
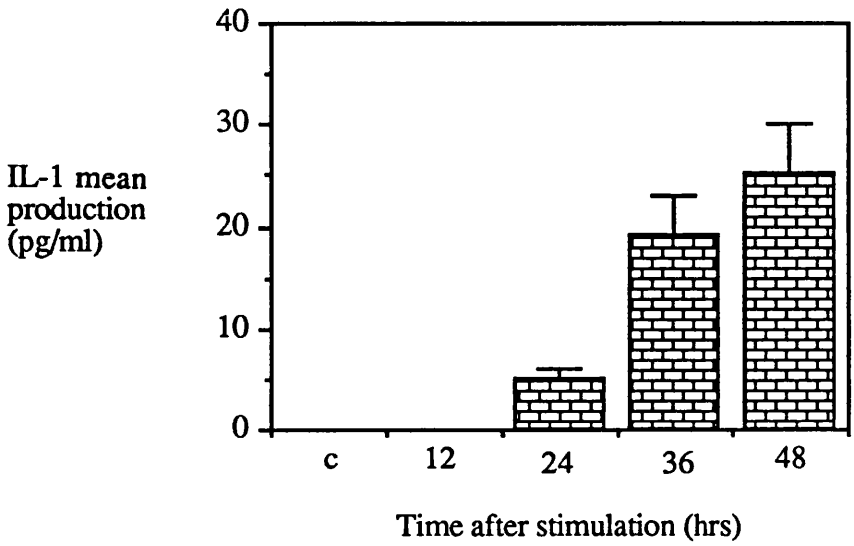


Figure IV.7. Induction of IL-1β mRNA by TNFα in human osteoblast-like cells.

Total RNA was extracted from cells as described in Materials and Methods. 5ug aliquots were loaded into the top slot of the manifold, subsequent vertical slots represent two fold dilutions of the RNA sample.

Lane 1 and 6, untreated cells at 6 and 24 hours. Lanes 2, 3, 4, and 5, treatment with 17ng/ml TNFα for 6, 8, 12 and 24 hours respectively. All lanes were normalized with a β-actin probe as shown in the panel below.



n = 6

Figure IV.8: TNFα stimulation of the production of IL-1 activity by human osteoblast-like cells.

Cell supernatants were assayed for IL-1 bioactivity at 12, 24, 36, and 48 hours after stimulation with 17ng/ml TNFα. The results represent assays in duplicate of triplicate treatment supernatants. The letter c represents untreated cells after 48 hours in culture. The vertical bars represent standard errors.

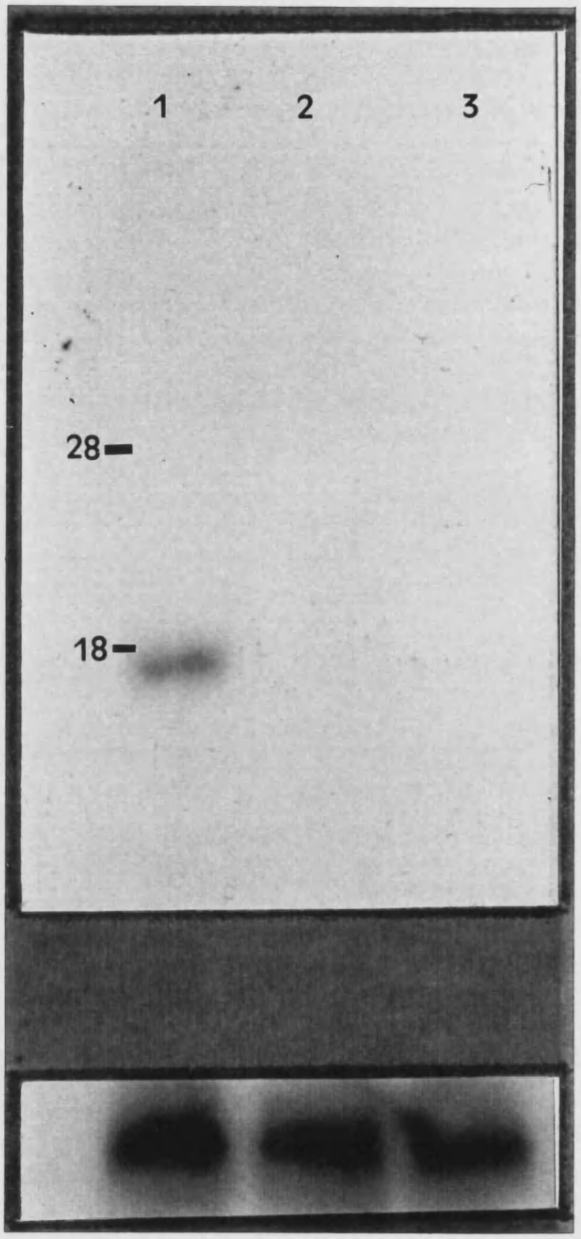


Figure IV.9. The effect of hydrocortisone on IL-1β mRNA expression.

Northern blot analysis was carried out on total RNA prepared as described in Materials and Methods, ten micrograms of total RNA was loaded on each lane.

Lane 1, treatment with IL-1α (200pg/ml) for 6 hours. Lane 2, treatment with both IL-1α (200pg/ml) and 10^{-8}M hydrocortisone for 6 hours. Lane 3, no treatment. All lanes were normalized using a β-actin cDNA probe, as shown in the panel below.

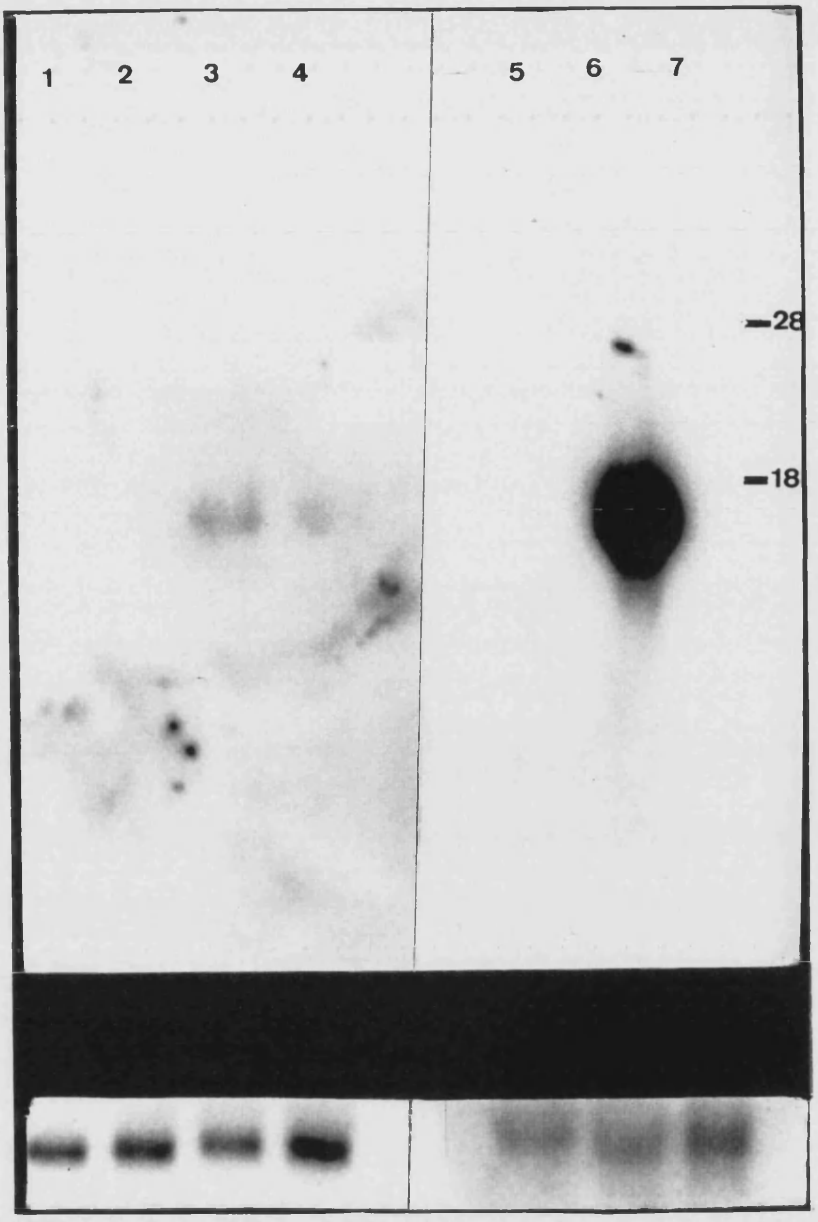


Figure IV.10. Lack of expression of IRAP mRNA by human osteoblast-like cells.

Northern blot analysis was carried out on total RNA prepared as described in Materials and Methods, ten micrograms of total RNA was loaded on each lane. Each section of the photograph represents 2 Northern blots which were hybridized together with the same IRAP probe.

Lane 1, human osteoblast-like cells stimulated with LPS. Lane 2, human osteoblast-like cells stimulated with PMA + LPS. Lane 3, PBMCs stimulated with PMA + LPS. Lane 4, PBMCs stimulated with LPS. Lane 5, untreated U937 cells. Lane 6, U937 cells stimulated with PMA + LPS. Lane 7, U937 cells stimulated with LPS.

All lanes were normalized using a β-actin cDNA probe, as shown in the panel below.

IV.6 DISCUSSION

This study confirms that human osteoblast-like cells are capable of expressing both IL-1β mRNA and IL-1 bioactivity, suggesting that IL-1 can be produced locally in the bone microenvironment without the presence of immune cells.

It was interesting to note that human osteoblast-like cells did not constitutively express IL-1β mRNA. This is in contrast to the expression of TGFβ mRNA and may reflect the fact that IL-1 is released in an active form as opposed to a latent peptide. Indeed, since IL-1 is such a potent resorptive agent it is not surprising that it is not constitutively expressed.

The amount of IL-1 bioactivity secreted by these cells was comparatively very low, since other cell types such as activated monocytes have been reported to express 10,000 times more IL-1 (Dinarello, 1988). However ,the low expression was not surprising when it is considered that even though mRNA was detected in the whole population, only about 5 % of the cell population seemed to be producing protein. This might be due to the fact that the immunolocalization technique was able to detect protein in the most highly producing cells only, although this seems unlikely. It has been reported in other cell types that the transcription of IL-1 can be initiated without detectable protein synthesis (Ikejina et al.1987), suggesting that there is some transcriptional factor which suppresses translation of the protein. It is possible that in the heterogeneous osteoblast-like cell population, there are some cells which have overcome this suppression, perhaps due to a differing stage of differentiation.

The perinuclear staining pattern of IL-1β observed in human osteoblast-like cells was unexpected, since it has been shown that IL-1 does not contain a leader sequence. IL-1 has been proposed to be secreted via a novel pathway as opposed to the classical route through the endoplasmic reticulum and Golgi apparatus (Rubartelli et al.1990). The localized staining pattern might be due to an intracellular pool of IL-1, as IL-1 has been shown to be present in membrane bound vesicles in the cytoplasm of monocytes

(Rubartelli et al.1990). It seems likely that a potent cytokine such as IL-1 would be localized in some manner, rather than dispersed throughout the cytoplasm.

The induction kinetics observed for IL-1β mRNA were very similar to those reported in different cell types. Fenton et al. (1988) reported that in the monocyte cell line THP1, LPS-induced IL-1β mRNA was very labile, and had a short half life of approximately 4 hours. LPS has been shown to increase the transcription of IL-1β mRNA in a specific manner. There is a period of short lived transcription followed by a sharp decrease in transcription. Over a longer time course transcription is subsequently initiated and the mRNA levels are again elevated. A similar transcription profile was seen with the human osteoblast-like cells. TNFα treatment gave rise to a sharp increase in IL-1β mRNA which quickly fell to basal levels. Unfortunately the experiments were not extended to determine whether a second peak of transcription occurred. The instability of IL-1β mRNA is thought to be due to an AU rich sequence in the 3 prime untranslated region (Shaw and Kamen, 1986). An AUUUA motif has been postulated to act as the recognition sequence for a processing pathway, specifically designed for the degradation of mRNAs for certain cytokines and protooncogenes.

The stimulus for IL-1β mRNA induction was quite specific, only "inflammatory" type cytokines seemed to initiate the response. TGFβ treatment over a 24 hour time course did not give rise to any induction of IL-1β mRNA, under conditions which had previously been shown to stimulate osteoblast proliferation. This response is cell type specific, since TGFβ has been shown to induce IL-1β gene expression in human monocytes within 2 hours of treatment (Chantry, 1992). The ability of cytokines to initiate different responses in specific cell types makes them ideal cell : cell signalling factors.

The identification of IRAP as a specific inhibitor of IL-1 activity in monocytes prompted the search for its expression in other cell types. Other inhibitors including TIMP and plasminogen activator inhibitor (PAI) are synthesized by many connective tissue cell types (Meikle et al.1991; Andreasen et al.1986). Therefore it was thought

that human osteoblast-like cells might synthesis IRAP. However the expression of IRAP seems to be more selective, since a number of different treatments failed to induce its expression in human osteoblast-like cells.

In agreement with similar studies, hydrocortisone completely suppressed the expression of IL-1α–induced IL-1β mRNA. Studies with the myelomonoblastic leukeamic cell line U937 showed that glucocorticoids markedly decrease IL-1β mRNA by two mechanisms (Lee et al.1988). In addition to inhibiting transcription, glucocorticoids selectively decrease the stability of IL-1β mRNA. It is thought that this destabilizing effect is achieved by the selective induction of specific RNAses. It is interesting to note that *in vivo* IL-1 induces ACTH release, and hence adrenal corticosteroid synthesis thereby creating a negative feedback loop on its own synthesis. IL-1 was shown in this system, in common with others (Warner et al.1987), to induce its own mRNA. These two observations seem to contradict each other, however autoinduction could take place in the bone microenvironment as opposed to a systemic down-regulation via corticosteroids.

In summary the expression profile for IL-1β mRNA in human osteoblast-like cells was completely different to that of TGFβ (see chapter III). Expression was induced by the "inflammatory" cytokines and LPS, but not influenced by the systemic hormones 1,25 D_3, PTH, 17-β oestradiol and hydrocortisone, whilst the direct opposite was observed for TGFβ.

It is tempting to speculate how these basic differences in expression may be relevant to the control of bone remodelling. Since IL-1 is such a potent resorbing agent it would seem preferable for its expression to be stimulated by agents which give a short transient signal, as opposed to a long lasting systemic signal. For this reason it is obvious that cytokines would be ideal signalling molecules for IL-1 mediated resorption. IL-1 itself could act as a very efficient signal, since it is not constitutively expressed, has a short mRNA half-life and has been shown to autoinduce its own mRNA in human osteoblasts. In contrast it seems more likely that the control of TGFβ

expression by the systemic growth hormones reflects its role in a less acute stage of remodelling, perhaps bone formation. Therefore it would seem from these studies that IL-1 expression is modulated by short transient signals, while TGFβ expression is regulated over a longer time course by the systemic growth factors.

Chapter V: The expression of TGFβ and IL-1β receptors by human osteoblast-like cells.

V.1 ABSTRACT

The presence of TGFβ and IL-1β receptors on human osteoblast-like cells was investigated. This was achieved by analysing the binding of fluorescent labelled ligands, by fluorescent activated cell sorting (FACS).

Mink lung cells which had previously been shown to express high levels of TGFβ receptors, were used to optimize a quantitative FACS analysis system. The number of TGFβ receptor sites per cell were estimated by calibrating the system with beads labelled with increasing numbers of fluorescein isothiocyanate (FITC) molecules.

Human osteoblast-like cells were shown to express receptors for both these cytokines. The binding of both cytokines to the cell surface was shown to be specific, since competition was demonstrated with unlabelled ligands. Both receptors were demonstrated to be trypsin sensitive, since trypsin treatment markedly decreased ligand binding.

Two populations of TGFβ receptors were putatively identified. They were shown to be present in high numbers of approximately 36,000 and 9,000 sites per cell. It was not possible to estimate the numbers of IL-1β receptors, but the low levels of fluorescence detected suggested that they were present in much lower levels.

Human osteoblast-like cells were treated with a range of cytokines and systemic hormones to determine whether these factors modulated the expression of TGFβ and IL-1β receptors. None of the factors tested were shown to modulate TGFβ binding over an 18 hour treatment period. IL-1β binding also remained unchanged after treatment with the systemic hormones 1,25 D_3, PTH, hydrocortisone and 17-β oestradiol. However treatment of the cells with the cytokines IL-1β, TNFα and LPS altered IL-1 binding. Both IL-1β and LPS treatment resulted in a modest decrease in IL-1β binding over a period of 18 hours. TNFα treatment increased IL-1β binding by approximately 40% over the same time period.

V.2 INTRODUCTION

There is much recent data to suggest that locally produced cytokines are involved in the control of bone remodelling. In previous chapters the expression of two important osteotropic cytokines, TGFβ and IL-1β has been described. It is very probable that the osteotropic activities of many cytokines are mediated via binding to specific cell surface receptors on osteoblasts. For this reason the occurrence of osteoblastic receptors for these two important cytokines, has been investigated.

Two different IL-1 receptors have been identifed, both are glycosylated proteins of 60kDa and 80kDa molecular weight. The expression of the 80kDa form is widespread, and its presence on many different cell types including fibroblasts (Solari, 1990), T cells (Kilian et al.1986), and various connective tissue cells (Bird and Saklatvala, 1986) has been demonstrated. The occurrence of the 60 kDa receptor seems to be more restricted but it has been identified on B cells (Matsushima et al.1986a, Horuk et al.1987). Both receptors have been shown to bind both IL-1 forms (Dower et al.1985) as well as the receptor antagonist IRAP (Granowitz et al.1991). The IL-1 receptor complex is rapidly internalized following IL-1 binding, which may be a necessary step for its mode of action.

The 80kDa receptor has been cloned (Sims et al.1989) from a murine cell line. Analysis of the predicted amino acid sequence shows that the protein consists of 3 distinct domains. The extracellular portion consists of 319 amino acids, and contains 3 immunoglobulin-like subdomains. A stretch of 21 hydrophobic amino acids has been identified, and postulated to be a transmembrane section. A large cytoplasmic domain of 217 amino acids has also been identifed.

The cDNA sequence of the 60kDa receptor has also been recently reported (McMahan et al, 1991). Analysis of the predicted amino acid sequence suggests that its structure is closely related to the 80kDa receptor. The ligand binding portion is simlar to the 80kDa form, and comprises of 3 immunoglobulin-like subdomains. A single hydrophobic transmembrane region has also been identified. Unlike the larger receptor, the 60 kDa

receptor contains a very short 29 amino acid cytoplasmic domain. It has been postulated that this difference in intracellular domain size may highlight a difference in signalling mechanisms between the two receptors.

There has been much controversy over the signal transduction mechanism for IL-1. Two different mechanisms have been proposed which have been reviewed recently by O'Neil et al. (1990) and Mizel, (1990). Mizel presented evidence that IL-1 induces a transient increase in cAMP levels via increased adenylate cyclase activity. The activation of adenylate cyclase is proposed to involve the interaction with a GTP binding protein since the process was shown to be pertussis toxin sensitive.

O'Neil et al. (1990) report that, in agreement with other groups they cannot detect any increase in cAMP levels after IL-1 binding. The first detectable signal seen following IL-1 binding was a rapid change in protein kinase activity. Two phosphorylation changes were identified in fibroblasts, both of which involved the phosphorylation of a serine residue. The first phosphorylation substrate was the EGF receptor. The second phosphorylation identified was that of a small heat shock protein hsp 27, which was phosphorylated within minutes of IL-1 stimulation. Since these phosphorylation events seem to be closely associated with IL-1 stimulation, O' Neil et al. postulate that IL-1 signal transduction involves the phosphorylation of a unique protein kinase.

Three TGFβ receptors have been identified termed type I, II and III, of 53kDa, 80kDa and 280-330kDa molecular weight respectively. The gene sequences of the Type II and III receptors have been recently obtained by mammalian expression cloning (Wang et al.1991, Lin et al.1992). Both are glycosylated proteins, with over 50% of the type III receptor (also called betaglycan) consisting of glycosaminoglycan moieties (Segarini and Seyedin, 1988). The binding of the 5 different TGFβ isoforms to these three receptors has not been fully characterised, but a complex network of cross reactive ligands and receptors is thought to exist (Cheifetz et al.1987).

The type II receptor has been shown to have a particularly high affinity for TGFβ1 although it also binds the other isoforms. It consists of a cysteine rich extracellular

domain, linked to a single hydrophobic transmembrane sequence. The intracellular transduction mechanism is unknown, but a putative serine/threonine kinase domain has been identified on the cytoplasmic portion of the protein (Lin et al.1992).

The type III receptor is an 853 amino acid protein which contains a large N terminal extracellular domain, a transmembrane region and a 41 amino acid cytoplasmic tail (Wang et al.1991). A soluble variant lacking the cytoplasmic domain has been shown to be secreted by some cell lines (Andres et al.1989). This receptor has been shown to be very abundant on the cell surface, and it is estimated that as many as 200,000 molecules may be present on the surface of a single cell (Cheifetz et al.1988). Due to the fact that it is so abundant and has a 10 fold lower binding affinity than the type I and II receptors, it has been postulated that it does not transduce an intracellular signal but rather acts as a cell surface reservoir for TGFβ.

There are a small number of reports that osteoblasts express both IL-1 and TGFβ receptors. Primary mouse osteoblast cultures have been shown to specifically bind radioiodinated IL-1 (Bird and Saklatvala, 1986). Shen et al. (1990) completed a more in depth study of primary mouse osteoblasts by Scatchard analysis, and showed that they expressed 3000-15000 receptors per cell with a dissociation constant of 30-200 pM. Various osteosarcoma cell lines including Saos-2/B10, MG-63, and UMR-106 (Bird and Saklatvala, 1986, Rodan et al.1991) have also been shown to express high affinity IL-1 receptors.

Wakefield et al. (1981) investigated the occurrence of TGFβ receptors in a range of cell lines. They identified high affinity receptors in every one of the 30 cell lines studied. Therefore it is probable that osteoblasts express TGFβ receptors, and there have been some preliminary reports to suggest that this is the case (Roberts et al.1987).

This chapter describes the design of a semi quantitative method to assess specifc cytokine binding to human osteoblast-like cells. These primary cultures are a heterogenous population of cells, containing different cell types including osteoblast precursors, pre-osteoblasts and fibroblasts. A popular method for characterising cell

surface receptors is Scatchard analysis of radioiodinated proteins. Unfortunately it is very difficult to interpret the data from such experiments, as the cells are not a homogeneous population. The overall ^{125}I-ligand binding observed represents the average binding of the whole population, and therefore it is possible to obtain artefactual results. In the case of TGFβ receptors there is the additional complication that the three different receptors are often present on the same cell. This makes the interpretation of Scatchard analysis very difficult. For these reasons a FACS analysis system was designed to estimate specific cytokine binding. This method has the advantage that the fluorescence of single cells can be determined, therefore distinct receptor positive and negative populations can be identified. This method was used to identify the presence of IL-1β and TGFβ receptors on human osteoblast-like cells. The ability of various systemic hormones and cytokines to modulate receptor expression has also been investigated to determine whether the osteotropic activities of these factors are mediated via receptor mediated events.

V.3 METHODS

Fluorescent activated cell sorting (FACS)

To obtain a satisfactory FACS analysis it was imperative that a single cell suspension was obtained. For that reason several different protocols were used to try to obtain a homogeneous single cell preparation of human osteoblast-like cells. This proved to be very difficult for many reasons. The main problem was that these cells are very adherent to each other and to tissue culture plastic. Also in the six weeks it takes for the cells to approach confluency, there is a large amount of matrix synthesised to which the cells adhere. Initially the cells were trypsinized from the 9 cm^2 petri dishes and seeded in non-tissue-culture grade petri dishes overnight. This gave quite a homogeneous non adherent cell preparation, but there were still large aggregates of cells present. Another protocol involved harvesting the cells from the primary cultures,

seeding the cells at a lower density ($5x10^5$/9cm^2 petri dish), and harvesting the cells for FACS analysis by trypsinisation. This protocol proved to be unsuitable as the trypsinisation resulted in a loss of ligand binding (see results section). The method which proved to give the best cell viability and binding was as follows, and was adopted for all subsequent experiments. The cells were trypsinized from the confluent primary cultures and seeded at a density of $5x10^5$cells/9 cm^2 petri dish. The cells were then settled overnight before treatment. The following agents were used in isolation to determine whether they affected receptor expression:

IL-1β (200pg/ml), LPS (500ng/ml), TGFβ (25ng/ml), TNFα (17ng/ml), 17-β Oestradiol (10^{-7}M), Hydrocortisone (10^{-8}M), 1,25 dihydroxyvitamin D_3 (10^{-8}M) and PTH (10^{-7}M). All treatments were undertaken for 18 hours unless stated for a particular experiment.

The treatments were carried out in serum-free media (1xMEM, 0.1% BSA, 10ug/ml transferrin, 1ug/ml insulin, $3x10^{-8}$M sodium selenate, 2mM glutamine, 100ug/ml penicillin /streptomyocin). After treatment, the cells were washed in PBS followed by PBS/ 2mM EDTA and harvested with a cell scraper. The cells were subsequently washed in RDF1 (a commercial buffer containing PBS and BSA), pelleted in a bench centrifuge at 1000 rpm for 5 mins, and resuspended in RDF1 at a cell density of 2×10^7/ml. 25 ul aliquots of the cell suspension were then dispensed into Becton Dickinson FACS tubes, which were kept on ice to prevent cell clumping. 10ul of biotinylated TGFβ (10ug/ml) or phycoerythrin conjugated IL-1β (IL-1β-PE) (6.5ug/ml) was then added to the cells and left to bind at 4°C for 30 min in the dark. Excess reagent was then removed by washing the cells in RDF1. For TGFβ staining, 10ul of avidin conjugated FITC reagent (10ug/ml) was then added for 30 min at 4°C in the dark. Excess reagent was removed by washing the cells with RDF1 buffer 3 times to prevent high background readings. The cells were finally resuspended in 0.5 ml RDF1 and immediately analysed on a FASCSTAR PLUS analyser. Alternatively the cells

were fixed to prevent a loss of ligand binding, by adding an equal volume of 2% paraformaldehyde/PBS and stored at 4°C.

Double labelling protocol

When the binding of both ligands to the same cells was investigated, a slighlty different protocol was used. The method was the same as previously described, but both ligands were added in the first 4°C incubation step. Excess ligand was removed using the same washing procedure.

Competition binding assays

It was necessary to demonstrate that the binding of the two ligands to the human osteoblast-like cells was specific. One method of verifying this specificity was to demonstrate competition for fluorescent ligand binding by unlabelled ligand. Since it was not possible to obtain large amounts of purified IL-1β and TGFβ, it was necessary to reduce the volume of cell suspension in the competion assays. This was achieved by incubating 10ul of cell suspension with 4ul of either IL-1β-PE or biotinylated TGFβ in LP22 tubes. 50 fold or 100 fold excesses of unlabelled IL-1β or TGFβ were added to the LP22 tubes, to compete with IL-1β–PE or TGFβ–FITC binding respectively.

Assessment of cell viability during FACS analysis.

It was considered necessary to assess the viability of the osteoblast-like cells during FACS analysis, as dead cells can often give rise to aberrantly high fluorescence values as they bind FITC molecules non specifically on their membranes. Propidium iodide was used to distinguish between dead and live cells. This fluorescent dye intercalates with double stranded nucleic acids to produce red fluorescence, proportional to the nucleic acid content, when excited by blue light. The dye is unable to pass through intact cell membranes, however when the cell dies and the integrity of the cell

membrane fails, it is able to enter and stain the nucleic acids. For this reason this dye was used to discriminate between live non-fluorescent cells and dead fluorescent cells. The cells were stained with propidium iodide as follows. Immediately prior to FACS analysis, a solution of propidium iodide (2.5mg/ml in PBS) was added to give a final concentration of 25ug/ml. The sample was then analysed using both the green and red fluorescence channels (FL 1 and FL 2 respectively). Figure V.1 shows a diagramatic representation of the cytogram of red versus green fluorescence that was produced. The dead cells were recognised as those with a high FL 2 values, and a gate was set so that they were not analysed any further.

It was not possible to undertake this type of analysis for the IL-1β binding studies, because the propidium iodide greatly interfered with the red fluorescence of the phycoerythrin labelled IL-1β. However over 20 FACS experiments were completed and the procedure was optimised so the proportion of dead cells was not greater than 15% in any case.

Analysis of data

The FACS data was analysed using the program LYSIS II, which gave mean fluorescence intensities (MFIs) for the cell populations in each treatment group. Each treatment group contained two duplicates. These fluorescence values were converted to receptor sites per cell (see results section) for the TGFβ binding studies. Unfortunately it was not possible to determine the number of IL-1β receptor sites per cell, as no calibrated phycoerythrin conjugated beads were commercially available, therefore the fluorescence values are reported as MFIs. The Students t Test was used to determine whether the treatments gave statistically different binding to the untreated cells.

V.4 RESULTS

Mink lung cells which have previously been shown to express TGFβ receptors were used to optimise TGFβ ligand binding. The degree of non-specific binding of the avidin FITC reagent was determined as shown in fig V.2a, where negligible background fluorescence was observed. When the mink lung cells were incubated with both biotinylated TGFβ and subsequently avidin FITC, a sub-population of the cells were seen to be markedly more fluorescent with an 8 fold increase in fluorescence intensity. A fluorescence gate was set (dotted line), for analysis purposes, and it was estimated that 35% of the mink lung cells bound the TGFβ. The TGFβ binding characteristics of human osteoblast-like cells were then investigated in separate experiments as shown in figure V.3. The background fluorescence obtained from the non-specific binding of avidin FITC to human osteoblast-like cells is shown in fig V.3a. Two positive TGFβ binding populations were identified (fig V.3b). One population consisting of 61% of the cells was only weakly fluorescent, with a 3 fold increase in fluorescence intensity. The other population seen as the second peak in fig 3b consisted of 39% of the whole population, and was highly fluorescent with a 23 fold shift in fluorescence intensity. The two populations were shown to have the same forward scatter, and side scatter characteristics (data not shown). Therefore the differences in fluorescence intensity observed, were not as a result of different size or granularity properties of the two populations of cells.

IL-1β binding was optimised using the $D_{10}N_4M$ helper T cell line which has been shown to express IL-1 receptors. There was a modest degree of binding of IL-1 to both $D_{10}N_4M$ cells (fig V.4c), and human osteoblast-like cells (fig V.4b). Using the fluorescence gate shown (dotted line) 30% of the human osteoblast-like cells were shown to bind phycoerythrin labelled IL-1β. The positive cells showed a 20 fold increase in fluorescence intensity.

The binding of both TGFβ and IL-1β to the human osteoblast-like cells was shown to be specific, since competition was demonstrated with unlabelled ligands. It can be seen from figure V.5a that treatment of the cells with 50 fold excess of unlabelled TGFβ, results in a marked reduction in biotinylated TGFβ binding. A similar result was seen for IL-1β binding (fig V.5b), in this case a 100 fold excess of unlabelled ligand was seen to decrease IL-1β binding to near background levels.

Both receptors were shown to be trypsin sensitive. Trypsin treatment reduced the binding of both ligands to a level approaching background fluorescence (fig V.6a and V.6b).

The TGFβ binding was quantified using commercially available FITC labelled beads. These beads gave 6 different peaks of fluorescence as shown in Fig V.7a. The MFI value for each peak was proportional to the degree of FITC labelling of the bead. Figure V.7b shows the linear relationship between fluorescence and log FITC equivalent per bead. This graph was then used to quantify the number of TGFβ molecules bound to each cell. The bimodal fluorescence profile of TGFβ binding was analysed using this calibrated system. It was estimated that the 2 fluorescence peaks corresponded to receptors at a density of 30,000 and 9,000 sites per cell.

An attempt was made to analyse the binding of both TGFβ (FITC labelled) and IL-1β (phycoerythrin labelled) to human osteoblast-like cells by using different fluorescence channels. Using this system it was hoped to characterise the binding of both ligands to the same cells. Unfortunately this approach did not prove possible, as shown in figure V.8. Positive TGFβ binding was demonstrated using the green FL 1 fluorescence channel when both ligands were present (fig V.8b). Additionally positive IL-1β binding could be demonstrated using the red FL 2 fluorescence channel when the cells were incubated with IL-1β-PE alone. However when both biotinylated TGFβ, and IL-1β-PE were incubated with the cells, no fluorescence above background level could be detected on the FL 2 channel (fig V.8e). This suggested that the biotinylated TGFβ was interfering with, or masking the IL-1β binding.

The human osteoblast-like cells were treated with a range of systemic hormones to determine whether they modulated TGFβ and IL-1β binding. None of the systemic growth factors used (including 1, 25 D_3, hydrocortisone, PTH and 17-β oestradiol) had any effect on either TGFβ or IL-1β binding (figs V.9 and V.10). TGFβ binding also remained unchanged after treatment with a range of cytokines including IL-1β, TGFβ and TNFα, (fig V.11). Often two populations of positive cells were seen to bind TGFβ (see figs V.4, V.7a and V.8a), the number of TGFβ binding sites for each population was estimated. Neither the proportion of each population, or the degree of TGFβ binding was affected by any of the treatments. However treatment of the cells with the cytokines IL-1α, and TNFα resulted in an alteration of IL-1β binding (fig V.12). Both IL-1β, and LPS treatment reduced IL-1β binding to approximately 70% of the basal level. TNFα treatment gave the opposite effect and increased IL-1β binding by 40% over the same time period.

V.5 FIGURES

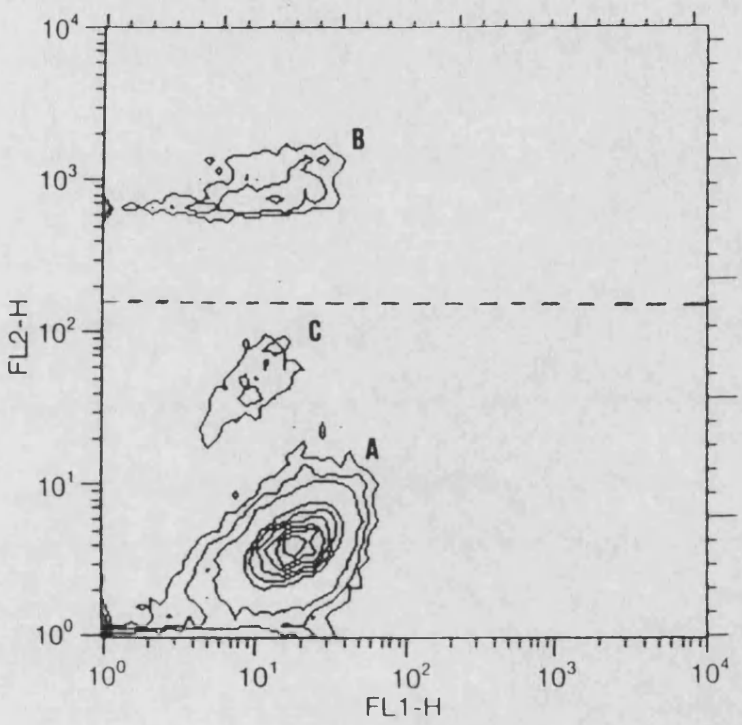


Fig. V.1 The use of propidium iodide for dead cell gating. Cells were incubated with 25ug/ml propidium iodide and a cytogram of red versus green fluorescence produced. The cluster of cells marked A are live, those marked B are dead, and those marked C are demonstrating a degree of breakdown in membrane integrity. A fluorescence gate was set as shown (dotted line), to exclude dead propidium iodide stained cells from subsequent analysis. The green fluorescence (FL-1) is a result of staining with a cell surface marker.

Fig. V.2 TGFβ binding by mink lung cells.

(a) shows the fluorescence profile obtained by analysing mink lung cells incubated with the avidin FITC reagent alone. There is a neglible level of non-specific background fluorescence (MFI = 3).

(b) Mink lung cells were incubated with both biotinylated TGFβ and avidin-FITC reagents. Thirty five percent of the mink population stained positively representing an 8 fold increase in fluorescence intensity.

Fig. V.3 TGFβ binding by Human osteoblast-like cells.

(a) Human osteoblast-like cells were incubated with avidin-FITC reagent alone. There was a neglible degree background fluorescence (MFI=3).

(b) Human osteoblast-like cells were incubated with biotinylated TGFβ and subsequently avidin-FITC. A bimodal fluorescence profile was observed, representing two populations of positive cells. The first fluorescent peak consisting of 61% of the whole population, was weakly fluorescent with a 3 fold increase in fluorescence intensity. The second peak consisting of 39% of the population was highly positive with a 23 fold increase in fluorescence intensity.

FIG 2

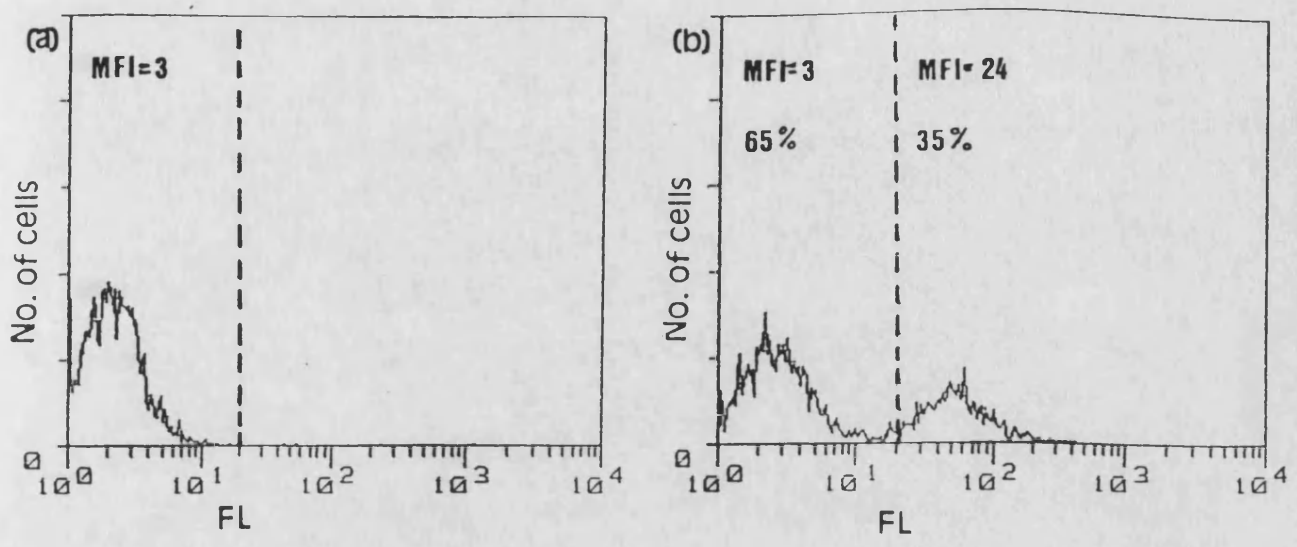

(a)
MFI=3
No. of cells
0
10^0 10^1 10^2 10^3 10^4
FL
(b)
MFI=3
65%
MFI=24
35%
No. of cells
0
10^0 10^1 10^2 10^3 10^4
FL

FIG3

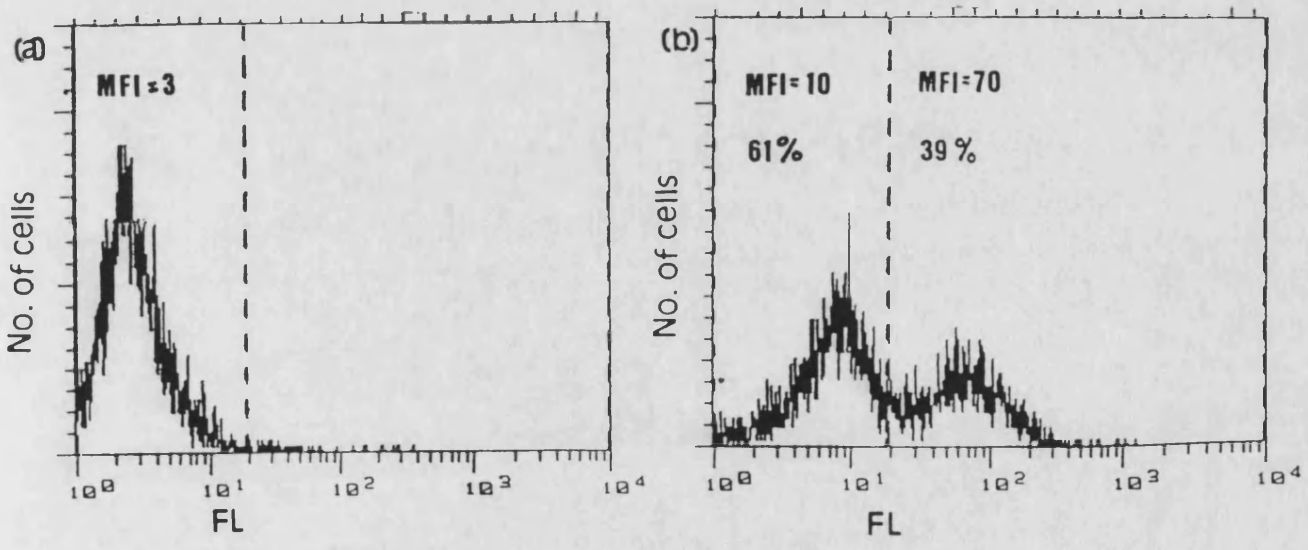

(a)
MFI=3
No. of cells
10^0 10^1 10^2 10^3 10^4
FL
(b)
MFI=10
61%
MFI=70
39%
No. of cells
10^0 10^1 10^2 10^3 10^4
FL

Fig V.4 IL-1β binding to $D_{10}N_4M$ and human osteoblast-like cells.

(a) Human osteoblast-like cells were incubated with PBS alone. A degree of background fluorescence was observed (MFI=15), due to the autofluorescent nature of the cells.

(b) Human osteoblast-like cells were incubated with phycoerythrin labelled IL-1β. A bimodal fluorescence profile was observed. Thirty percent of the human osteoblast-like cells were seen to be positively fluorescent with a 20 fold increase in fluorescence intensity.

(c) The murine, IL-1 receptor positive T helper cell line ($D_{10}N_4M$), was incubated with phycoerythrin labelled IL-1β. A small proportion of the cells (25%), were seen to be positively fluorescent. Note the fluorescence profile of $D_{10}N_4M$ cells incubated with PBS alone (data not shown), was analysed, and was very similar to the profile shown in V.4a.

FIG 4

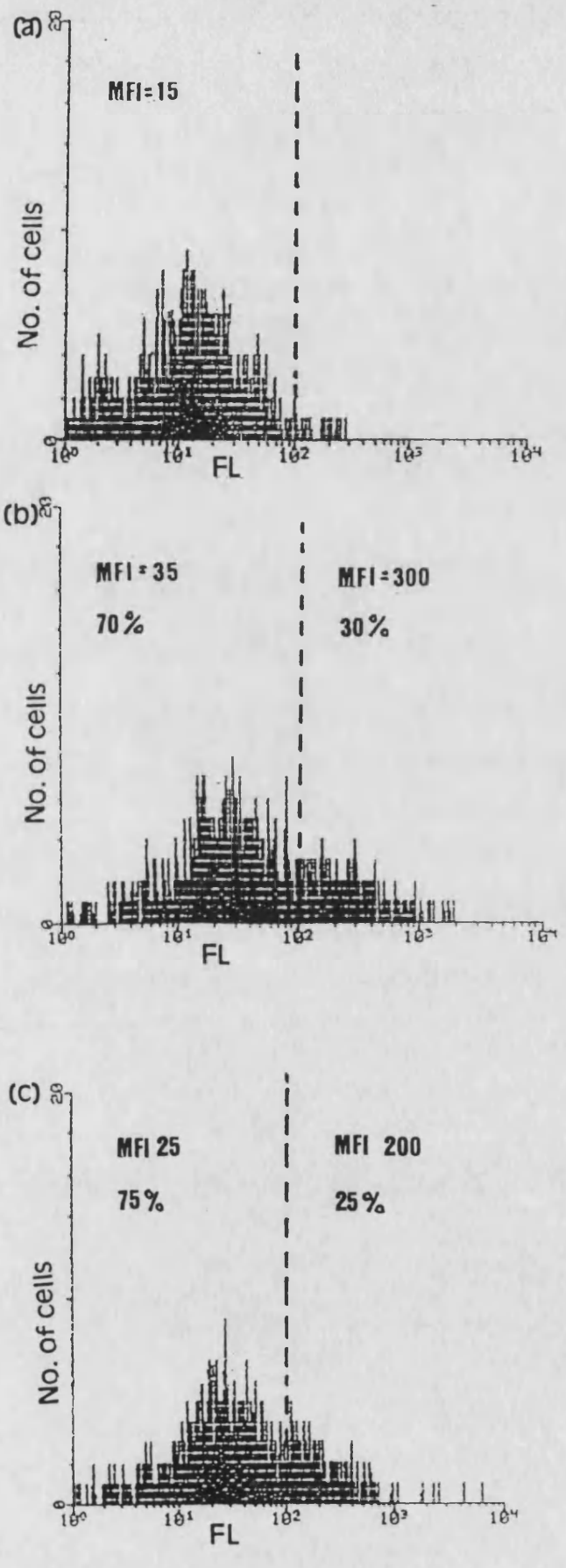

(a)
MFI=15
No. of cells
FL
(b)
MFI = 35
70%
MFI=300
30%
No. of cells
FL
(c)
MFI 25
75%
MFI 200
25%
No. of cells
FL

Fig. V.5a Competition of biotinylated TGFβ binding by unlabelled TGFβ.

Non-specific background fluorescence (shaded red) was determined by analysing human osteoblast-like cells incubated with avidin-FITC alone.

A bimodal fluorescence profile (shaded grey) was obtained by analysing human osteoblast-like cells incubated with biotinylated TGFβ and subsequently avidin FITC.

The effect of excess unlabelled TGFβ on biotinylated TGFβ binding, was determined by analysing human osteoblast-like cells incubated with biotinylated TGFβ plus a 50 fold excess of unlabelled TGFβ, and subsequently avidin FITC. The fluorescence profile (shaded green) can be seen to decrease to a level approaching that of background fluorescence.

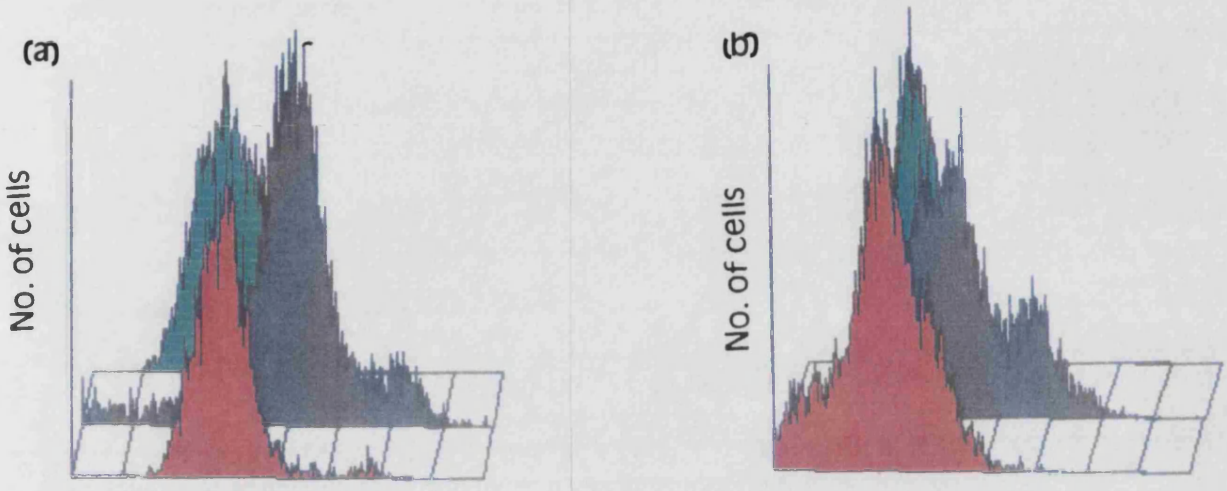
Figure V.5b Competition of phycoerythrin labelled IL-1β binding by unlabelled IL-1β.

Non-specific background fluorescence (shaded red) was determined by analysing human osteoblast-like cells incubated with PBS alone.

The fluorescence profile (shaded grey) was obtained by analysing human osteoblast-like cells incubated with phycoerythrin labelled IL-1β. By setting a fluorescence gate containing 95% of the negative cells, it was estimated that approximately 30% of the whole population were positive.

The effect of excess unlabelled IL-1β on IL-1β-PE binding, was determined by analysing human osteoblast-like cells incubated with phycoerythrin labelled IL-1β plus a 100 fold excess of unlabelled IL-1β. The resulting fluorescence profile (shaded green) can be seen to decrease to a level approaching that of background fluorescence.

FIG 5



avidin- FITC MFI = 60
Bio-TGFβ + avidin-FITC MFI = 254
Bio-TGFβ + avidin-FITC MFI = 75
+ 100 fold XS TGFβ

PBS MFI = 20
IL-1β-PE MFI = 88
IL-1β-PE MFI = 24
+ 100 fold XS IL-1β

Fig. V.6a Effect of trypsin treatment on TGFβ binding.

Non-specific background fluorescence (shaded red) was determined by analysing human osteoblast-like cells incubated with avidin-FITC alone.

The profile (shaded grey) was obtained by analysing human osteoblast-like cells incubated with biotinylated TGFβ and subsequently avidin FITC. Two fluorescent populations of human osteoblast cells are seen.

The effect of trypsin was determined by analysing human osteoblast-like cells treated with trypsin prior to incubation with biotinylated TGFβ, and subsequently avidin FITC. Trypsin treatment can be seen to markedly decrease TGFβ binding.

Fig. V.6b Effect of trypsin treatment on IL-1β binding.

The red fluorescence profile was obtained by analysing human osteoblast-like cells incubated with PBS alone, and represents background fluorescence.

The grey fluorescence profile was obtained by analysing human osteoblast-like cells incubated with phycoerythrin labelled IL-1β. By setting a fluorescence gate containing 95% of the negative cells it was estimated that approximately 30% of the whole population were positive.

The effect of trypsin was determined by analysing human osteoblast-like cells treated with trypsin prior to incubation with phycoerythrin labelled IL-1β. The fluorescence profile (shaded green) shows that the trypsin treatment caused a marked decrease in IL-1β binding.

FIG 6

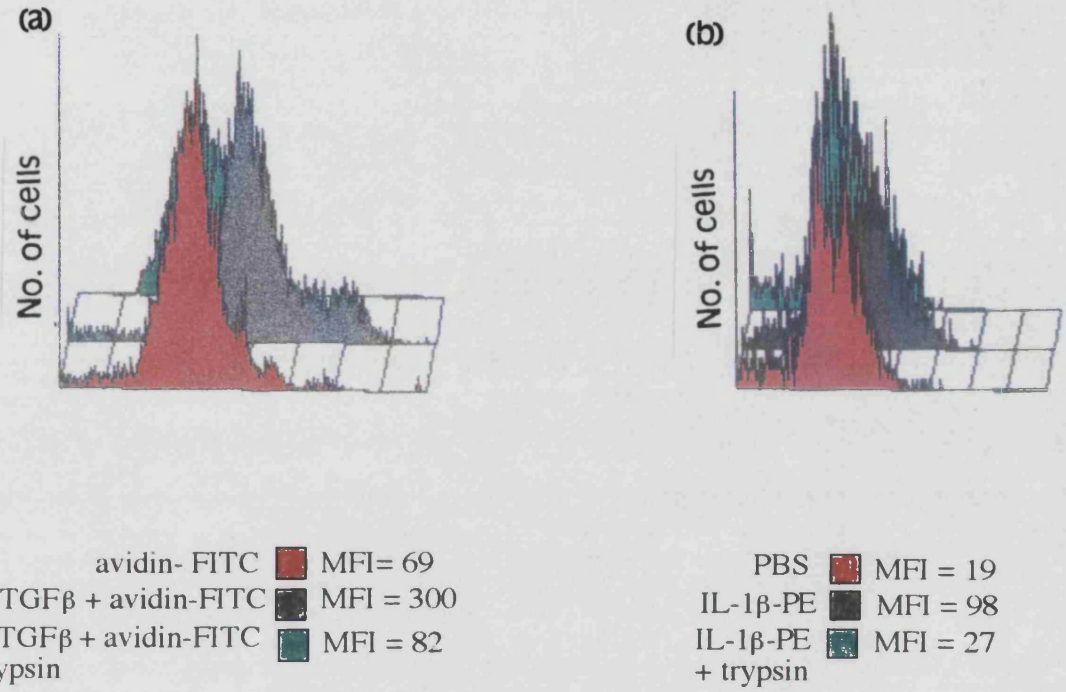
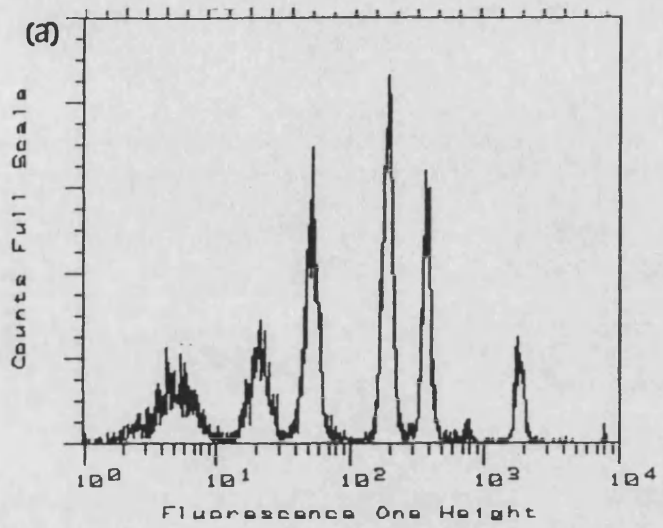

(a)
No. of cells
avidin- FITC
MFI= 69
Bio-TGFβ + avidin-FITC
MFI = 300
Bio-TGFβ + avidin-FITC
+ trypsin
MFI = 82
(b)
No. of cells
PBS
MFI = 19
IL-1β-PE
MFI = 98
IL-1β-PE
+ trypsin
MFI = 27

Fig. V.7 Calibration of the FACS analyser with FITC labelled beads.

(a) The fluorescence profile of a mixture of commercially available beads with increasing numbers of FITC molecules per bead was analysed. The profile obtained, consisted of 6 different peaks, corresponding to the 6 different bead populations. Six fluorescence gates were set and a MFI value obtained for each peak.

(b) A graph of the peak MFI vs the log FITC equivalent. It can be seen that the MFI for each peak was proportional to the degree of fluorescence. This linear relationship was used to quantify TGFβ ligand binding, by estimating the number of FITC labelled binding sites per cell.

FIG 7


(a)
Counts Full Scale
10^0
10^1
10^2
10^3
10^4
Fluorescence One Height

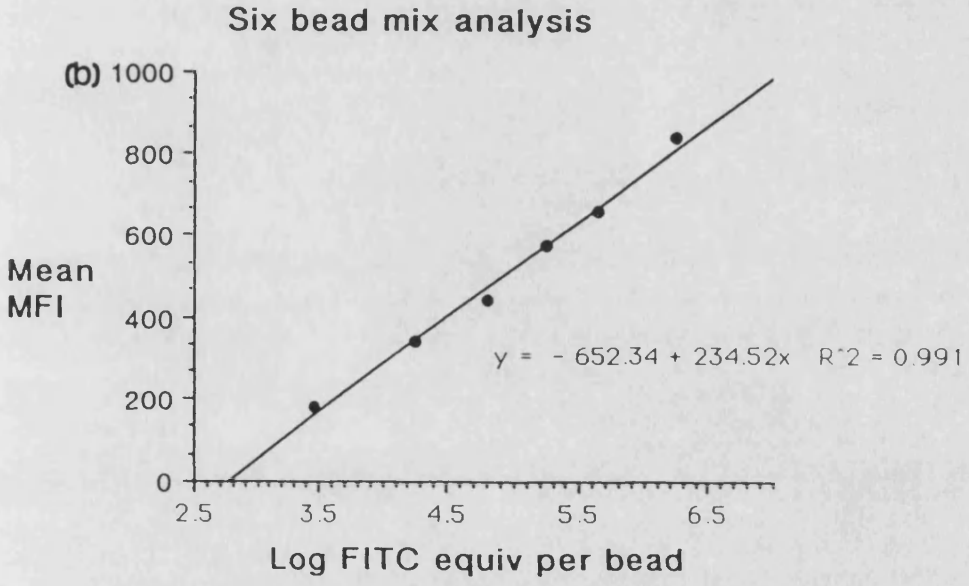

Six bead mix analysis
(b) 1000
800
600
400
200
0
Mean MFI
y = - 652.34 + 234.52x R^2 = 0.991
2.5
3.5
4.5
5.5
6.5
Log FITC equiv per bead

Fig. V.8 FACS analysis of double labelled cells.

(a) Background green (FL-1) fluorescence due to non-specific avidin-FITC binding

(b) Two positive FL-1 fluorescent populations were seen from an analysis of human osteoblast-like cells incubated with biotinylated TGFβ and phycoerythrin labelled IL-1β, and subsequently avidin FITC.

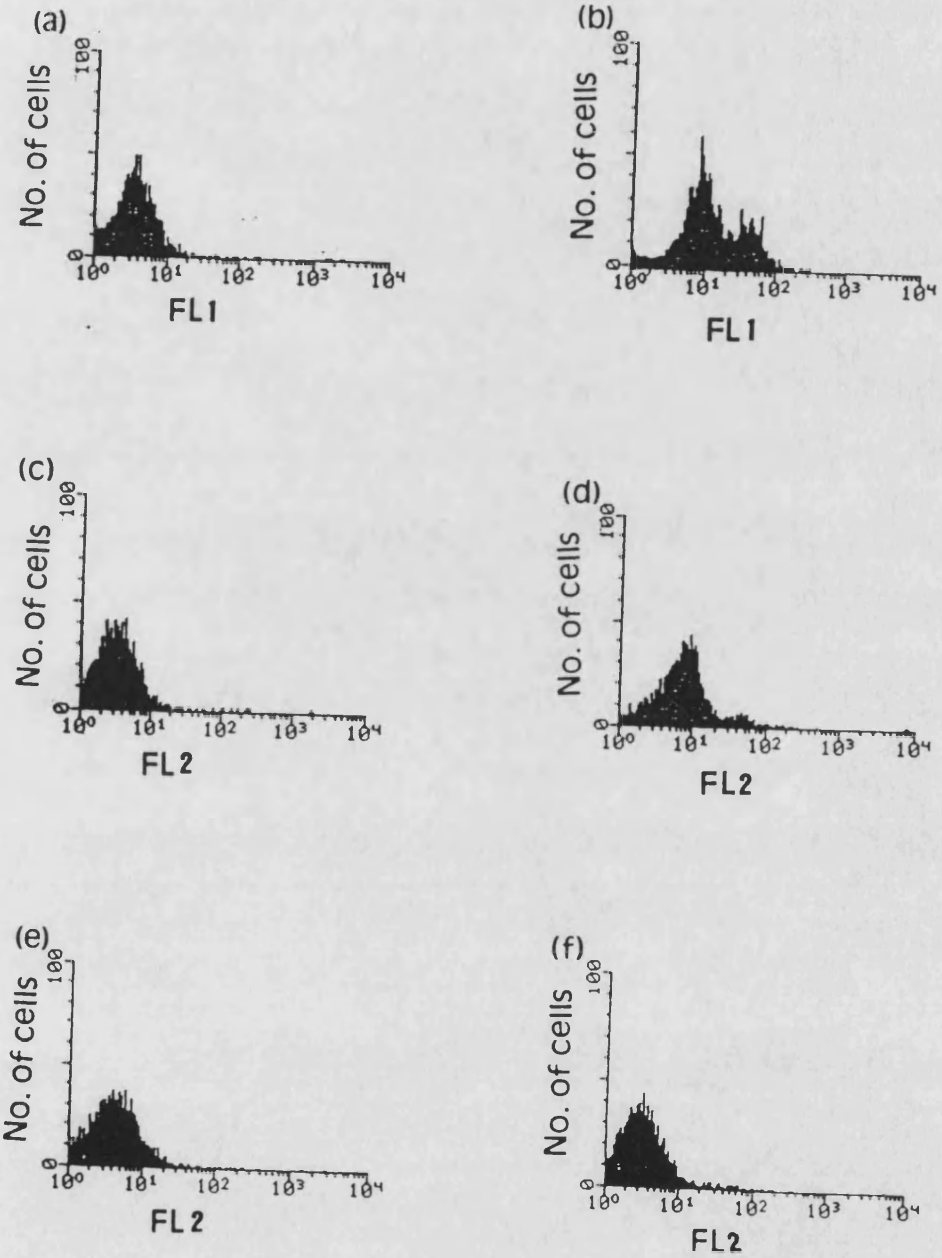
(c) FL-2 (red) fluorescence profile of human osteoblast-like cells incubated with PBS alone.

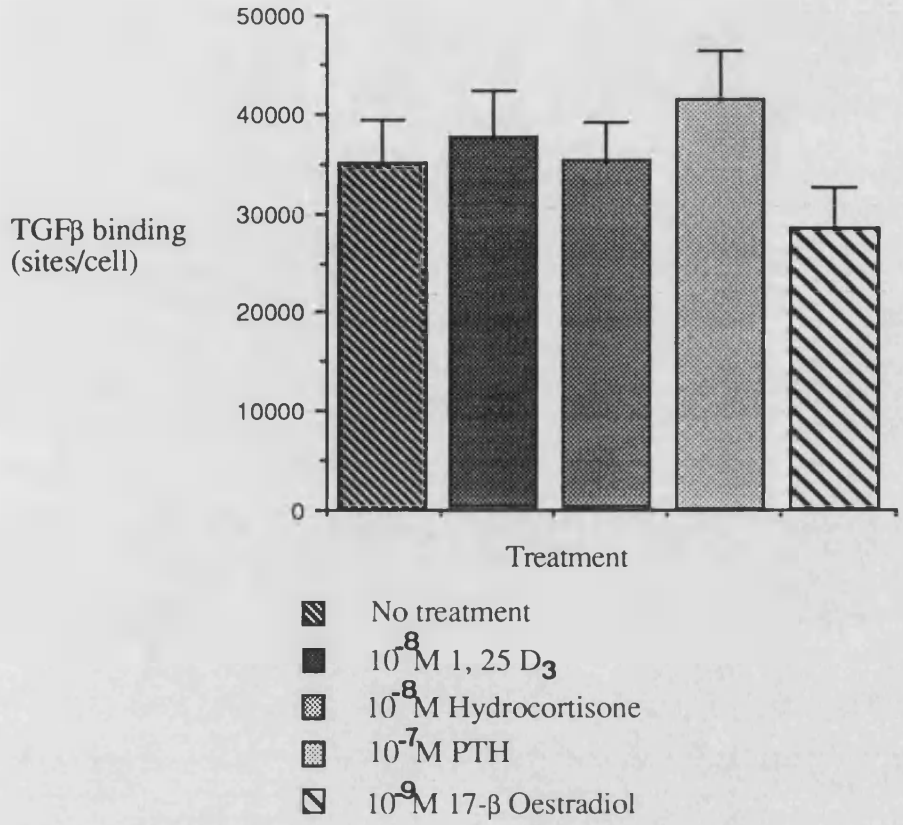
(d) A population of positive cells were seen on the FL-2 channel from an analysis of cells incubated with phycoerythrin labelled IL-1β alone.

(e) No FL-2 fluorescence above background levels was detected in cells incubated with biotinylated TGFβ and phycoerythrin labelled IL-1β, and subsequently avidin FITC.

(f) The fluorescence profile of cells incubated with biotinylated TGFβ and avidin FITC on the FL-2 channel. The profile is essentially negative, demonstrating that the green fluorescence was not "breaking through" into the red FL-2 channel

FIG8

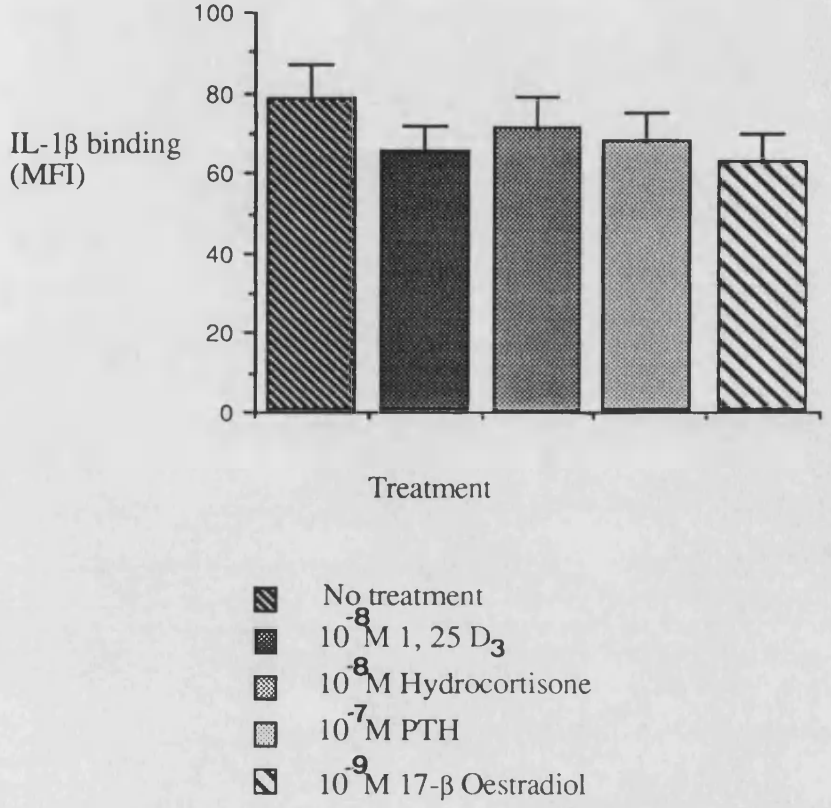

(a)
No. of cells
FL1
(b)
No. of cells
FL1
(c)
No. of cells
FL2
(d)
No. of cells
FL2
(e)
No. of cells
FL2
(f)
No. of cells
FL2



n = 4

Fig. V.9 The effect of the systemic hormones on TGFβ binding.

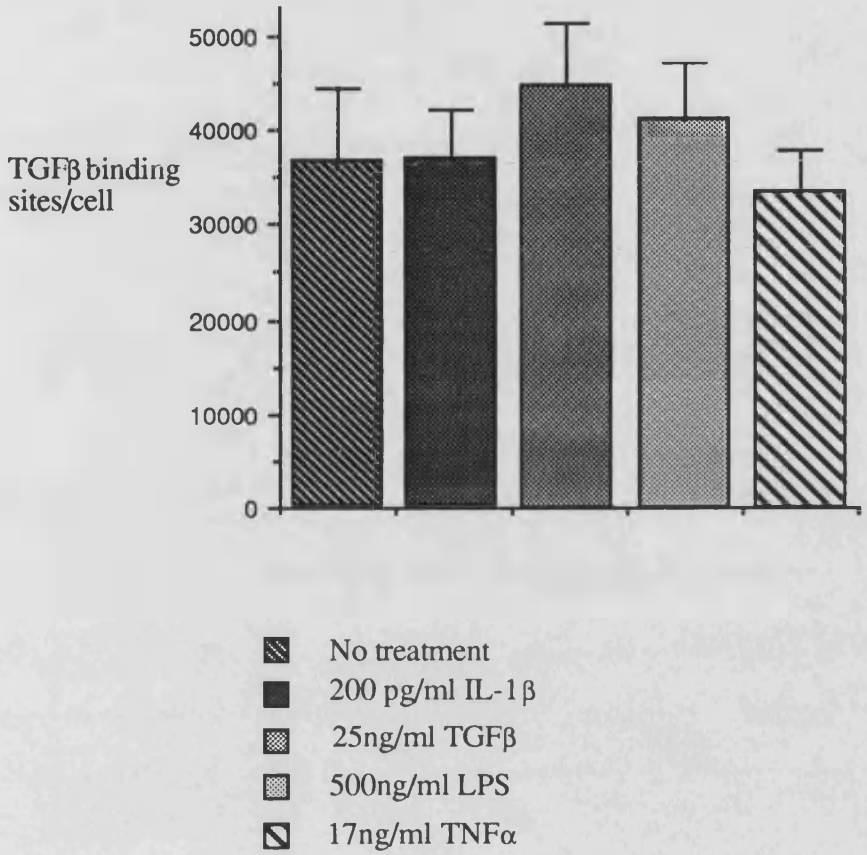
The human osteoblast-like cells were treated with each of the following factors in isolation for 18 hours at 37°C; 10^{-8}M 1,25 D_3, 10^{-8}M hydrocortisone, 10^{-7}M PTH or 10^{-9}M 17β oestradiol. FACS analysis of TGFβ binding was subsequently performed and the number of TGFβ binding sites were estimated as described in figure V.7. No significant change in TGFβ binding was observed following any of these treatments. Vertical bars represent standard errors.



n = 4

Fig. V.10 The effect of systemic hormone treatment on IL-1β binding by human osteoblast-like cells.

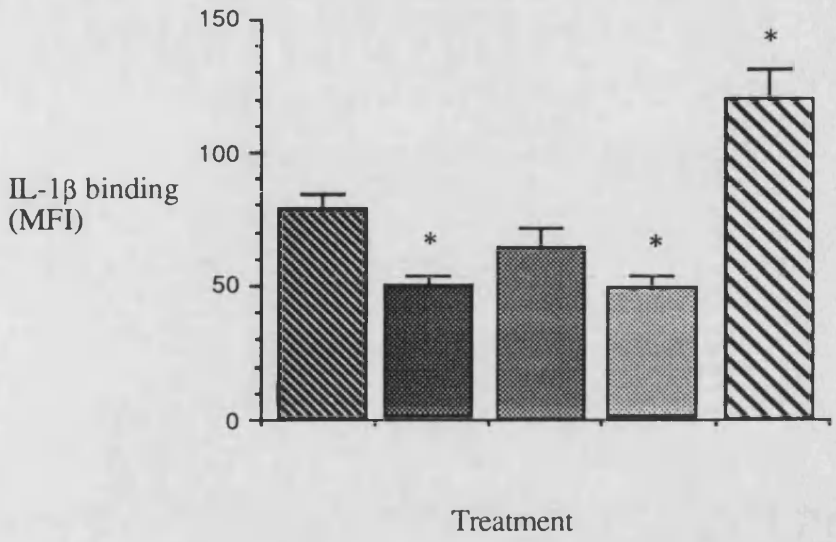
The human osteoblast-like cells were treated with each of the following factors in isolation for 18 hours at 37°C; 10^{-8}M 1,25 D_3, 10^{-8}M hydrocortisone, 10^{-7}M PTH or 10^{-9}M 17β oestradiol. FACS analysis of IL-1β binding was subsequently performed. No significant change in IL-1β binding was observed following any of these treatments. Vertical bars represent standard errors.



n = 4

Fig. V.11 The effect of cytokine and LPS treatment, on TGFb binding by human osteoblast-like cells.

The human osteoblast-like cells were treated with each of the following factors in isolation for 18 hours at 37°C; 200pg/ml IL-1β, 25ng/ml TGFβ, 500ng/ml LPS, and 17ng/ml TNFα. FACS analysis of TGFβ binding was subsequently performed. No significant change in TGFβ binding was observed following any of these treatments. Vertical bars represent standard errors.



n = 4

Fig V.12 The effect of cytokine and LPS treatment, on IL-1β binding by human osteoblast-like cells.

The human osteoblast-like cells were treated with each of the following factors in isolation for 18 hours at 37°C; 200pg/ml IL-1β, 25ng/ml TGFβ, 500ng/ml LPS, and 17ng/ml TNFα. FACS analysis of IL-1β binding was subsequently performed. IL-1β and LPS reduced IL-1β binding to 70% of basal levels. TNFα treatment over the same time course increased IL-1β binding by 40%.

A Students T test was undertaken to determine whether the differences were statistically significant. The degree of significance is shown above. Vertical bars represent standard errors.

V.6 DISCUSSION

This preliminary study has shown that human osteoblast-like cells possess cell surface binding sites for both TGFβ and IL-1β. It was interesting to note that routinely only about 30% of the human osteoblast-like cell population were shown to express these receptors. This observation might be explained by the idea that this subpopulation corresponds to a distinct osteoblast differentiation stage, or cell type.

Analysis of the bimodal TGFβ fluorescence profile suggests that two receptor populations are present. These two populations are estimated to exist on the osteoblast-like cells at densities of approximately 30,000 and 9,000 binding sites per cell. These values are of a similar magnitude to the number of TGFβ receptors estimated on mouse fibroblasts by radioligand binding (Wakefield et al.1981). The existence of a bimodal fluorescence profile could possibly be explained by the occurrence of different receptor binding affinites.

Even though no estimation of the number IL-1β binding sites could be undertaken, by examining the fluorescence profiles it is probably that human osteoblast-like cells express far fewer IL-1 receptors. This would agree with the findings of many studies which estimate that IL-1 receptor levels are fairly low on many different cell types (Bird et al.1988). Osteoblasts are not an exception to this observation, studies using radiolabelled IL-1β estimate that the IL-1 receptor density is approximately 3000 in primary cultures of mouse osteoblasts (Bird et al. 1986). The low cell surface density of IL-1 receptor and the rapid rate of internalization of the ligand-receptor complex, make this system exquisitely sensitive to low extracellular IL-1 concentrations. IRAP has been shown to inhibit several IL-1-induced effects *in vitro*, however a 10-100 fold concentration of IRAP over IL-1 is always required (Dinarello 1991). This suggests that a low degree of receptor occupancy is required to tranduce the IL-1 signal. It is possible that the small (30%) decreases in IL-1β binding that were observed may be biologically relevant if these factors are taken into account.

Both receptors were shown to be trypsin sensitive. An analysis of the predicted amino acid sequences of the TGFβ (Lin et al.1992, Wang et al.1991) and IL-1β (McMahan and et al, 1991, Sims et al.1989) receptors show that there are many putative trypsin sites. It has been suggested that a soluble form of the TGFβ III receptor may be formed by proteolysis at a trypsin site of the extracellular domain .

The large number of TGFβ receptors compared to the number of IL-1β receptors present on the osteoblast cell surface may explain why no results were obtained from the two colour fluorescence experiments. There are several possible reasons why no IL-1β binding was detected in the presence of TGFβ ligand. TGFβ might have preferentially bound to the cell surface, and made access for the IL-1β ligand difficult. This could be easily tested by incubating the cells firstly with the IL-1β ligand, then with the TGFβ ligand. It is more likely that the high levels of green fluorescence produced due to TGFβ binding masked the red signal from the IL-1β ligand. Before two colour fluorescence can be achieved, it is necessary to remove a percentage of the green fluorescence that breaks through the red fluorescence channel by setting compensation limits. For this reason it is possible that the low levels of red fluorescence expected were removed due to the compensation limits. This problem could be overcome by using phycoerythrin labelled TGFβ and FITC labelled IL-1β.

The number of TGFβ cell surface receptors did not seem to be modulated by any of the osteotropic factors studied. This suggests that the activities of these factors are not mediated by a change in the numbers of TGFβ receptors. There are several reports where the cytokine in question, but not its receptor is modulated by other cytokines and hormones (Littlewood et al.1991, Boumpas et al.1991). The fact that TGFβ receptors are present in high levels might make modulation unfeasible. Obviously the modulation of low numbers of receptors would be more easily achieved. Perhaps this is why IL-1β receptor levels were shown to be modulated by the treatment of certain cytokines.

There have been reports that TGFβ receptor levels are cell density dependent (Newman et al.1989). It might be interesting to address this question, although it is possible that this is a cell type specific phenomenon.

This preliminary study could form a basis for a more extensive evaluation of osteoblastic TGFβ and IL-1β receptors. It would be preferable to further validate this technique, by using cell lines that are known not to express IL-1β or TGFβ receptors. Such negative cell lines have been characterised for IL-1 receptors, but it would be very difficult to obtain a TGFβ negative cell line since most cells seem to possess these receptors.

Only one time point was used in this study, due to the fact that the experiments were very costly and required large numbers of human osteoblast-like cells from the same donor. The time point used (18 hours after stimulation), was chosen so that the data could be directly compared with the study of IL-1β receptors on mouse osteoblasts (Shen et al. 1990). However the results from previous studies (Littlewood et al.1991) and those reported in other chapters of this thesis highlight the fact that cytokines are often expressed in a transient manner by human bone cells. Therefore it is possible that the expression of the cell surface receptors for these factors may also be modulated at a specific time. Before it can be unequivocally stated that the receptors studied were not modulated by several osteotropic factors, it would be necessary to extend the study over a series of different time points.

Early time points might be particularly relevant since it is possible that receptor mediated events occur rapidly. Previous work suggests that IL-1 cell surface receptors are rapidly down-regulated following IL-1 treatment at 37°C. Matsushima et al. (1986b) detected a decrease in IL-1 binding as early as 1 hour after stimulation with IL-1. Therefore it would be interesting to extend this study to determine at which time points IL-1β binding decreases.

FACS analysis can only detect changes in the amount of ligand that is bound following different treatments. It is possible that the decrease in IL-1β binding observed

following treatment with LPS and IL-1β, could be due to a change in the affinity of the receptor for its ligand. Scatchard analysis of IL-1β binding would confirm this was the case. However as mentioned previously, it is very difficult to interpret the results of Scatchard analyses of mixed cell populations. One approach to over come this problem might be to sort the human osteoblasts into different cell populations. Presently this technique is not available, but with the advent of specific monoclonal antibodies for human osteoblast-like cells (Walsh et al.1991) it may be possible to select specific osteoblast populations by FACS in the future.

IL-1 production by human osteoblast-like cells following LPS stimulation was marginally detectable after 12 hours. It is possible that the decrease in IL-1β binding seen following LPS treatment could be due to LPS stimulating the production of IL-1. If the levels of IL-1 required for receptor down regulation are low, this may be a mechanism by which the inflammatory signal is dampened.

The observation that TNFα treatment gives rise to a small but reproducible increase in IL-1β binding cannot be explained by this mechanism. In a similar manner to LPS, TNFα treatment of human osteoblast-like cells also results in the production of IL-1 (see chapter IV). Therefore it was unexpected that TNFα treatment gave this contrasting result. TNFα and IL-1β are known to act synergistically in several systems (Stashenko et al 1985), possibly this upregulation of IL-1β binding may be a method of achieving synergy. TNFα could increase IL-1β binding to facilitate an increased IL-1 signal over a short time course. There is a precedent for this phenomenon, since it has been shown that IL-1 stimulation transmodulates the binding of the EGF receptor in fibroblasts (Bird and Saklatvala, 1989).

These FACS studies do not give any information about the type of receptor each ligand is binding to. To determine which type of IL-1 and TGFβ receptors are present on the cells it necessary to undertake some biochemical studies of the receptors. Two methods which give information about the molecular weight of the receptor are ligand blotting and cross linking experiments. The first technique, ligand blotting has been

successfully employed by several groups to determine the molecular weights of the two IL-1 receptors. It involves the purification of cell membranes which are subsequently solubilized to give a suspension of the protein components. The sample is then electrophoresed by PAGE to resolve the different protein species. The proteins are then transferred and fixed to a nitrocellulose membrane by Western blotting. The molecular weight of the receptor is then determined by incubating the membrane with radiolabeled ligand, and comparing the radioactive signal with protein markers.

It is probable that the 80kDa type I IL-1 receptor, would be the form present on human osteoblast-like cells, since it has been shown to be present on fibroblasts, and mouse osteoblasts (Shen et al.1990), and that the distribution of the 60kDa type seems to be more restricted. Using the technique outlined it would be possible to confirm this fact.

The second technique involves binding a radiolabelled ligand to the surface of the cell type in question, and then chemically crosslinking the receptor-ligand complex. This approach is particularly useful when the ligand binding capability of a receptor is impaired by solubilization and electrophoresis. The molecular weight of the complex is then determined by PAGE. Using these two techniques it might be possible to define which types of IL-1 and TGFβ receptors are present on the surface of human osteoblast-like cells.

It is particularly important to determine whether the cell surface binding of $TGF\beta_1$ is primarily due to the presence of type III TGFβ receptors. This type of receptor is not thought to tranduce a signal following TGFβ binding, and therefore the exclusive presence of this receptor would prohibit the autoinduction of TGFβ mRNA observed in this cell type (see chapter III).

The observations of this study agree with several of the findings of the Shen study. In that study preincubation with IL-1 reduced IL-1 binding, and the IL-1β ligand was shown to be rapidly internalized. TGFβ treatment was also shown to decrease the number of IL-1 binding sites (Shen et al. 1990). No TGFβ induced down regulation of IL-1β binding was seen in this study. It is possible that the effect is dependent on the

concentration of the stimulus, since TGFβ effects have been shown to be biphasic in many systems. The concentration of TGFβ used in this study, was greater than 10 fold higher than that used in the Shen study, this might explain the difference in the results. The Shen study also stated that PTH treatment increased the number of IL-1β receptors present on mouse osteoblasts by 50%, in disagreement with the results from this study. However they found, in agreement with this study, that the systemic hormones 1,25 D_3 and 17-β-oestradiol had no effect on IL-1 receptor levels. The differences in results between the two studies might be due to the occurrence of species specific phenomenon.

In summary, the demonstration that human osteoblast-like cells express cell surface receptors for the cytokines TGFβ and IL-1β, further supports the theory that local cytokine actions are important factors in the control of bone remodelling.

Chapter VI: TGFβ1 and IL-1β expression in human bone as detected by *in situ* hybridization.

VI.1 ABSTRACT

Recent evidence suggests that cytokines may play an important role in cell : cell signalling in bone remodelling. Work described previously in Chapters III and IV demonstrates that cultured human osteoblast-like cells are capable of expressing the cytokines TGFβ1 and IL-1β. These *in vitro* studies have been extended using *in situ* hybridization of RNA probes to decalcified cryostat sections of human bone, to investigate cytokine expression *in situ.*

The tissue for this study was taken from osteophytes of osteoarthritic femoral heads. It has been extensively characterised, and shown to contain areas of active bone formation and resorption.

TGFβ1 expression was detected in chondrocytes in the osteoarthritic cartilage. The expression was shown to be dependent on the differentiation stage of the cell, with immature chondrocytes expressing the most TGFβ1 mRNA. In contrast, in the same areas of cartilage no IL-1β mRNA expression was detected. Newly formed fibrocartilage cells at the periphery of the osteophyte also expressed high levels of TGFβ1 mRNA, but only weak IL-1β expression was detected. The osteoblasts laying down osteoid on woven bone surfaces expressed high levels of TGFβ1 mRNA. At areas of bone resorption large multinuclear cells reminicent of osteoclasts were shown to express high levels of TGFβ1 mRNA. The adjacent bone marrow tissue also contained cells expressing TGFβ1.

During intramembranous bone formation newly differentiated osteoblasts expressed high levels TGFβ1 mRNA. Additionally high IL-1β expression was detected at certain sites of intramembranous bone formation.

The expression of both cytokines diminished with the formation of mature lamellar bone, and was shown to be higher in active osteoblasts (apposed to osteoid) than in quiescent lining cells. Transient expression of both cytokines was detected during osteophyte closure, where the expression was shown to correspond to distinct stages of osteophyte maturation.

VI.2 INTRODUCTION

The expression of IL-1β and TGFβ *in vitro* by human bone cells has been discussed in chapters III and IV. In this chapter the research has been extended to characterise the expression of these factors in bone tissue. Such a study is important, since culture conditions are obviously very different from the bone microenvironment. Therefore the cytokine expression profiles observed *in vitro* might differ greatly with those *in vivo*. The following section documents what is known about the localization of TGFβ and IL-1β in bone tissue.

It is widely accepted that TGFβ is incorporated into the matrix of bone, where it is found in levels 200-fold higher than other tissues (Seyedin et al. 1985). Immunolocalization studies and different extraction techniques have been used to investigate the cellular association and tissue distribution of TGFβ in skeletal tissue. Ellingsworth et al. (1986) described the localization of a factor termed CIFA (which has been shown to be identical to $TGF\beta_1$) in fetal bovine tissues. CIFA/$TGF\beta_1$ staining was shown to be abundant in osteocytes throughout cancellous and cortical bone. A similar immunocytochemical study of fetal murine bone was undertaken by Heine et al. (1987). TGFβ staining was detected in osteoblasts during both endochondral and intramembranous ossification. Particularly intense staining was seen in the osteoblasts apposed to the membranous bone in the calvariae of 15 day old mice embryos. In the long bones during endochondral bone formation, intense staining of TGFβ was also seen in primary ossification centres. Areas of joint formation, such as articulations between vertebrae and long bones also contained high amounts of TGFβ.

A very different approach was used by Carrington et al. (1988), where the role of TGFβ in endochondral bone formation was monitored *in vivo*. Endochondral bone formation was induced in rats by the implantation of demineralised bone matrix. This system had been previously characterised, and it was shown that the type of bone formation in the resulting ossicle, was indistinguishable from normal endochondral

bone formation. At various times the implants were removed and extracted with a denaturing solution, and the TGFβ content quantified by a radioimmunoassay. The results showed that the TGFβ, which was present in the original bone matrix, was removed by macrophage-like cells. After 9 days newly synthesized TGFβ was detectable, and could only be extracted after demineralisation, which suggested it was tightly bound to the bone matrix. The TGFβ content of the ossicles was shown to increase until 21 days after implantation. The increase was shown to correlate with the onset of vascularisation and calcification of the cartilage. The ossicles were also removed, sectioned and stained with a TGFβ polyclonal antibody. Staining was detected initially in inflammatory cells, and later in chondrocytes and osteoblasts. The most intense staining of TGFβ was found in calcified cartilage and mineralised bone matrix, indicating a preferential compartmentalisation of TGFβ in mineralised tissue. This time dependent accumulation of TGFβ in cartilage during its transition to bone, suggested that TGFβ was an important factor in the regulation of ossification during endochondral bone development.

There are many studies which show that chondrocytes express TGFβ. Chondrocytes within the fetal bovine articular cartilage were shown to contain CIFA/TGFβ$_1$ although those within the growth plate were not stained (Ellingsworth et al. 1986). This finding was supported by Pelton et al. (1991) who monitored the expression of three TGFβ isoforms during fetal skeletal development. Chondrocytes in ribs and vertebrae showed strong TGFβ$_3$ staining, but only very weak staining for TGFβ$_1$ and TGFβ$_2$. TGFβ$_3$ staining in the chondrocytes was observed to remain high during chondrocyte maturation. Whilst TGFβ$_1$ and TGFβ$_2$ staining was evident with the development of hypertrophic chondrocytes. The idea that the TGFβ isoforms were expressed in a specific developmental manner was further supported by the finding that TGFβ$_1$ levels were especially high in the perichondrium, where very little TGFβ$_2$ or TGFβ$_3$ staining was seen.

A study using *in situ* hybridization techniques to monitor TGFβ and c-fos expression

in fetal bone development has been reported (Sandberg et al.1988). The initial findings of this study using Northern blot analysis suggested that TGFβ mRNA was enriched in the growth plate, a finding which is directly conflicting with the data of Ellingworth et al. (1986). The *in situ* hybridization of TGFβ probes to sections of the fetal human bone, confirmed that a select population of chondrocytes in the growth plate and epiphyses were expressing TGFβ mRNA. These conflicting reports of expression of TGFβ in the growth plate could highlight the difference between human and bovine fetal chondrogenesis, or more probably, the fact that the two stages of development studied were different.

Other bone cell types which have been shown to express TGFβ *in situ* include those in the bone marrow particularly platelet producing megakaryocytes (Pelton et al. 1991).

Despite the fact that IL-1 synthesis has been reported in osteoblasts from many species (Lorenzo et al.1990; Keeting et al.1991), there has been very little data presented documenting IL-1 expression in bone tissue. This may be due to the fact that IL-1 is rapidly turned over and therefore expression is difficult to detect. There has been a report of IL-1 expression in normal mouse bone (Takacs et al. 1988). *In situ* hybridization techniques were used to detect IL-1α mRNA expression in tissue sections of bone, although the cell types expressing this cytokine were not identified.

The results from many *in vitro* experiments suggest that both IL-1 and TGFβ are very important osteotropic cytokines. For this reason a study was undertaken to monitor the expression of these cytokines in human bone. It was thought that by investigating the expression of these factors *in situ*, it would be possible to understand how cytokines were modulated during human bone remodelling. Cryostat cut sections of human osteophyte tissue were hybridized with TGFβ1 and IL-1β RNA probes. Human osteophyte tissue was used for two reasons. Firstly, as it has been extensively characterised, and shown to contain abundant amounts of all the different bone cell types. Secondly, there are many areas of active bone remodelling within the tissue, in contrast with other bone tissues such as adult illiac crest where remodelling sites are

rarely detected. Serial sections were stained using cytochemical techniques for alkaline phosphatase and TRAP activity, two enzymes which are highly expressed by osteoblasts and osteoclasts respectively. Using this approach it was hoped to determine which cells were capable of expressing these cytokines, and to study the temporal expression of these factors during osteophyte development.

VI.3 MATERIALS AND METHODS

Preparation of tissue

Developing osteophytes were dissected from the femoral heads removed from patients undergoing hip arthroplasty for osteoarthritis. The tissue was dipped briefly into 5% (w/v) polyvinyl alcohol (PVA), and then chilled (-70°C) by precipitate immersion in cooled n-hexane (low in aromatic hydrocarbons grade, boiling range 67-70°C). Cryostat sections (10 um) of unfixed, undecalcified bone were cut on a modified Bright /Hacker cryostat equipped with a finely polished tungsten tipped steel knife. The knife was further cooled to -70°C by packing it's shaft with dry ice. The temperature of the cryostat cabinet was kept below -25°C. The sections were picked off the knife onto TESPA coated glass slides.

The *in situ* expression of $TGF\beta_1$, and IL-1β mRNA with corresponding negative controls (sense transcripts) was analysed in serial sections of each osteophyte and surrounding tissue, starting at the tip of each outgrowth and proceeding downwards in the direction of the subchondral bone to disclose the various stages of maturation. Fifteen osteophytes were analysed in five separate experiments. Corresponding histologies for each group of sections were stained with a modified Wrights stain.

Generation of RNA probes

The TGFβ1 RNA probe was generated by *in vitro* transcription from a 300 b.p. cDNA template contained in the plasmid pGHTGFB-27. The plasmid was digested with the restriction enzymes HindIII or EcoRI to generate two linear DNA templates containing

either SP6 or T7 RNA polymerase sites as shown in fig VI.1. Antisense transcripts were generated using SP6 RNA polymerase, and sense transcripts using T7 polymerase. Both RNA probes were assessed to confirm they were of the same specific activity (see chapter II).

IL-1β RNA probes were generated in a similar manner from a 350 b.p. cDNA template contained in the plasmid pgem2il1beta. The plasmid was digested with the restriction enzymes EcoR1 or Pvu II as shown in fig. VI.2, to give two linear templates containing T7 and SP6 RNA polymerase sites respectively. Antisense transcripts were generated by *in vitro* transcription using T7 RNA polymerase, and sense transcripts by using SP6 RNA polymerase.

In Situ Hybridization

The *in situ* hybridization of the TGFβ1 and IL-1β RNA probes was undertaken as described in Chapter II 4(iii).

Figure VI.1: The generation of TGFβ1 RNA probes.

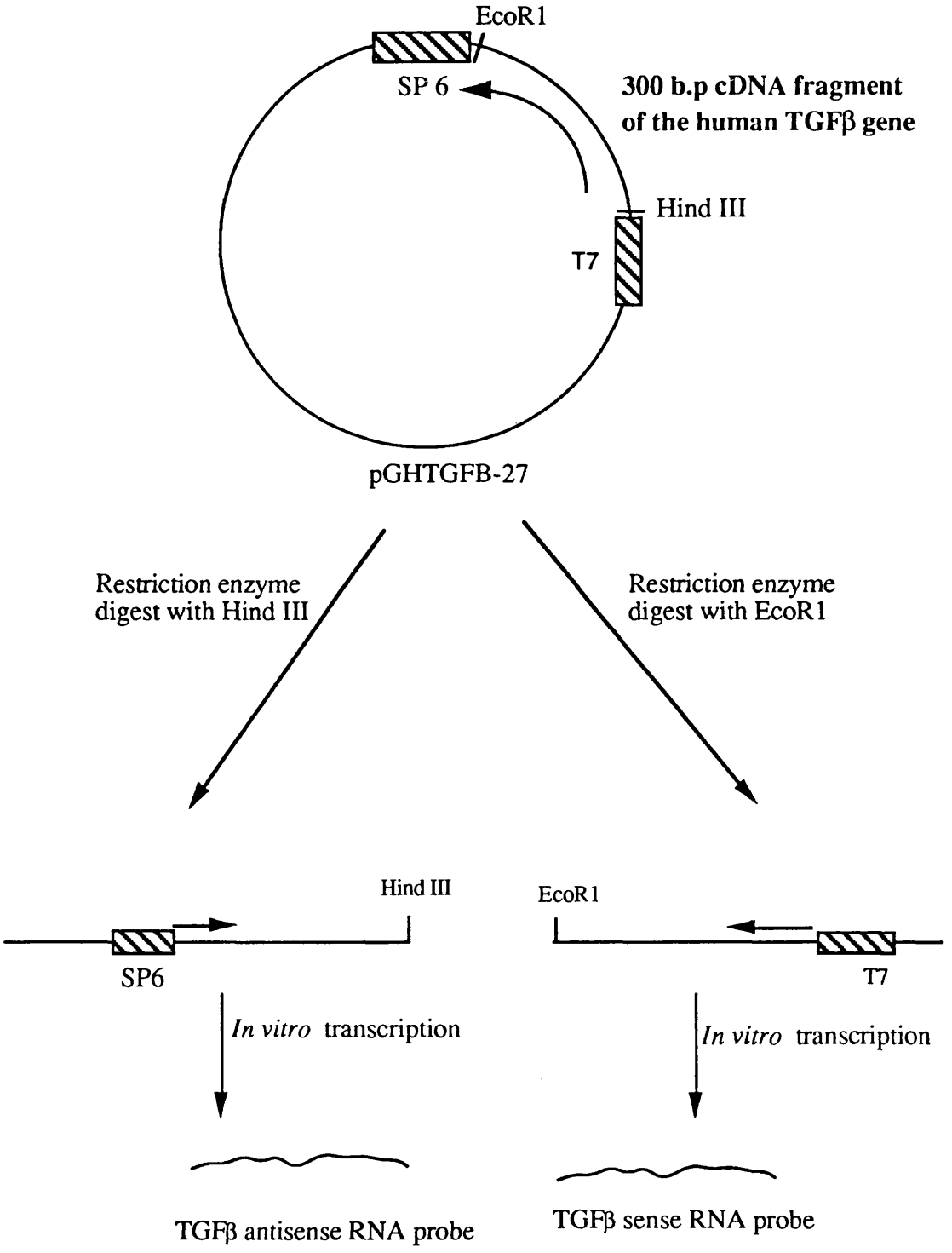


Figure VI.2 : The generation of IL-1β RNA probes.

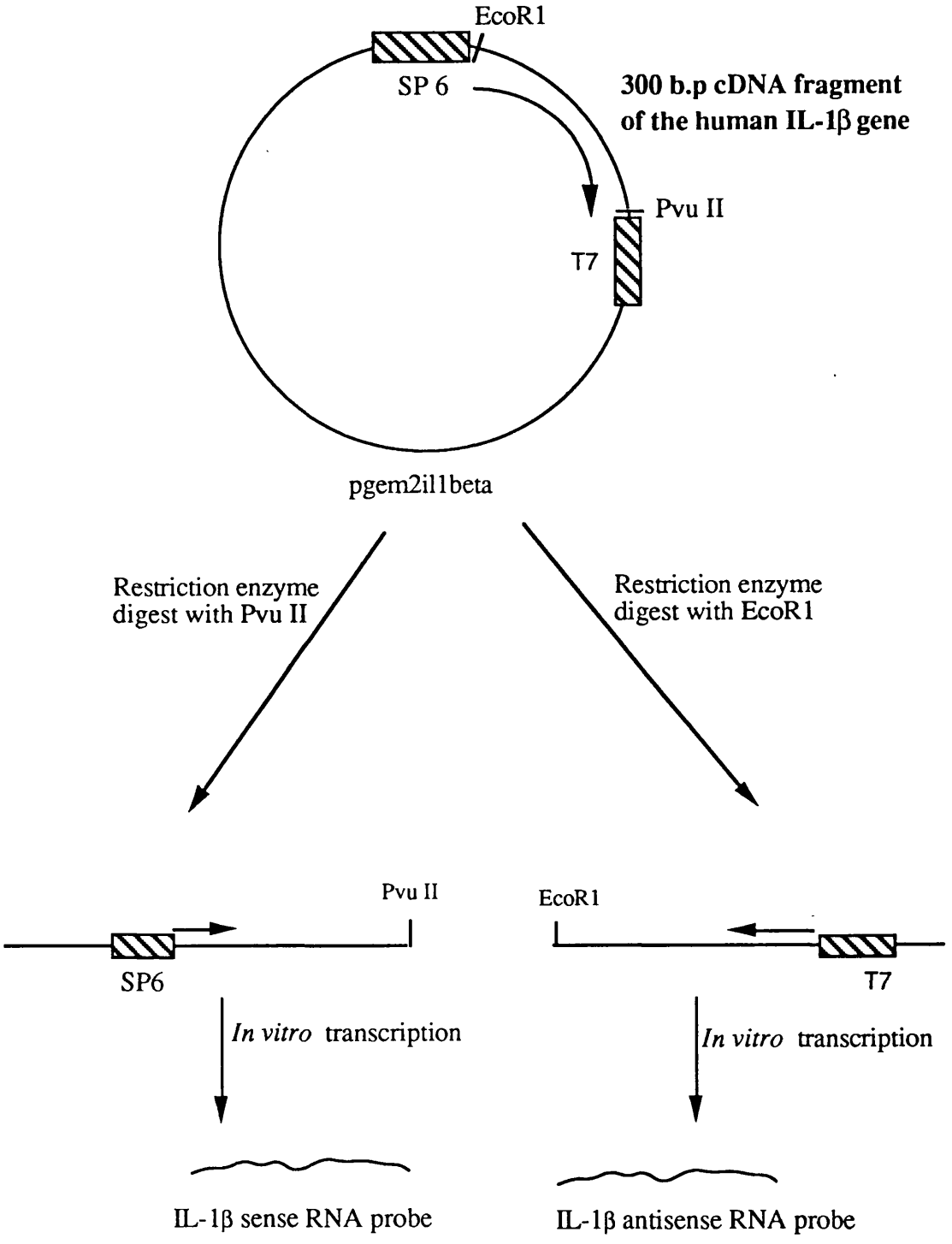
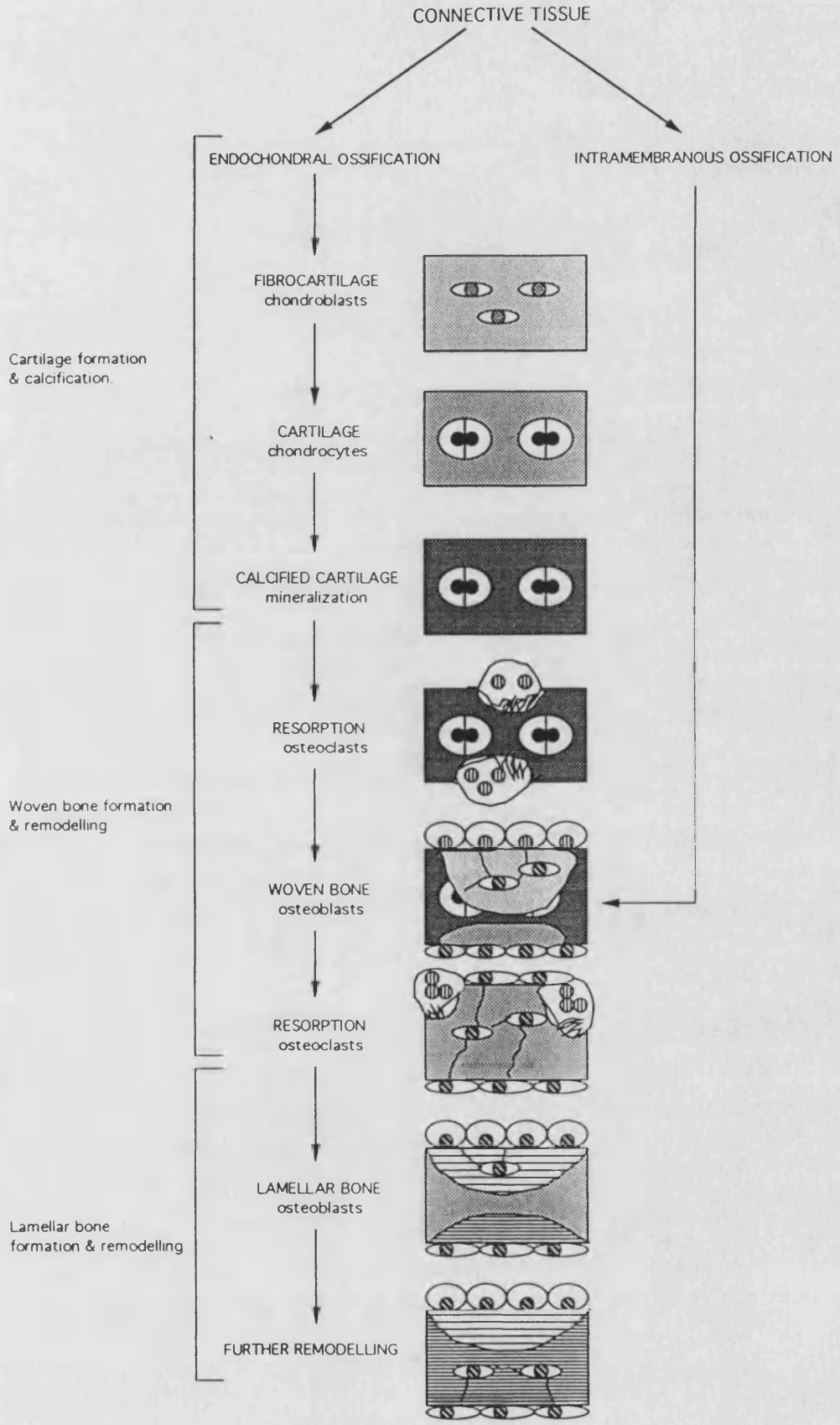


Figure VI.3: The three main stages of osteophyte development.



VI.4 RESULTS

The osteophyte tissue is very complex and has been extensively characterised (Dodds and Gowen, 1992). There are three main stages of osteophyte development; the initial cartilage formation and calcification stage, followed by woven bone formation and remodelling, culminating with the formation of mature lamellar bone. These stages of osteophyte development are depicted pictorially in fig VI.3.

CARTILAGE FORMATION AND CALCIFICATION

In certain regions of the osteophyte tissue there were recognisable areas of organized cartilage. Three zones of cartilage cells could be distinguished; proliferating and resting chondrocytes, hypertropic chondrocytes, and calcified cartilage (fig. VI.4a). The expression of TGFβ in these areas was shown to be dependent on the differentiation state of the chondrocytes. Newly formed chondrocytes expressed moderate levels of TGFβ mRNA, whereas the expression by hypertrophic chondrocytes was markedly lower. No expression was detected in the calcified cartilage zone (fig. VI.4b). Serial sections of the same area of cartilage tissue revealed that there was no IL-1β expression during the initial cartilage formation phase (fig. VI.4c).

The outermost layer of developing osteophytes consisted of newly formed fibrocartilage that had differentiated from the surrounding mesenchymal connective tissue (fig. VI.5a). Differentiating fibrocartilage cells showed strong TGFβ1 mRNA expression (fig. VI.5b), but only very weak expression of IL-1β (fig. VI.5c). Again the expression of TGFβ1 seemed to be dependent on the differentiation stage of the fibrocartilage cell. Mature fibrocartilage cells showed a distinct reduction in TGFβ1 mRNA expression compared with the more immature cells. In contrast IL-1β expression within the mature fibrocartilage cells remained at the same low level as in the immature cells.

BONE FORMATION.

Two distinct types of bone formation occur concurrently in the developing osteophyte, namely endochondral and intramembranous ossification.

As described previously (see chapter I.2), endochondral bone formation occurs on a cartilage template. This cartilage template is invaded by vascular connective tissue. After vascularization the cartilage is extensively remodelled by osteoclasts to form woven bone. Subsequently bone is layed down by osteoblasts on the residual cartilage.

No TGFβ1 or IL-1β expression was detected in osteoblasts apposed to the calcified cartilage surfaces during the initial phase of cartilage remodelling (data not shown). Further remodelling of the calcified cartilage / woven bone results in the formation of trabeculae of woven bone rich in osteoblastic surfaces replacing the cartilage. At these sites TGFβ1 expression was high in active osteoblasts apposed to thick seams of osteoid (fig. VI.6a). Again IL-1β expression was rare but discrete areas of expression were observed (fig. VI.6c).

In areas of newly formed intramembranous bone large osteoblasts could be seen to differentiate from the surrounding connective tissue and form woven bone directly without a cartilage template (fig. VI.7a). At these sites, differentiating osteoblasts demonstrated strong TGFβ1 mRNA expression (fig VI.7b and VI.7e). Occasionally high levels of osteoblastic IL-1β expression were detected in discrete areas of intramembranous bone formation (fig. VI.7c and VI.8b).

WOVEN BONE REMODELLING

Within sections further through the osteophyte blocks extensive osteoclastic remodelling of the bone was observed. The marrow between the trabeculae was highly cellular and filled with mononuclear stromal cells and migrating osteoclasts (fig VI.9a). Very occasionally certain populations of large multinucleated cells (resembling

osteoclasts) expressed TGFβ1 mRNA (fig. VI.9b)

LAMELLAR BONE FORMATION

Osteoblasts laying down the lamellar bone that replaced the resorbed woven bone (fig. VI.10a), expressed high levels of TGFβ1 mRNA. Higher expression was detected in the cuboidal active osteoblasts, and less expression within the flattened lining cells (fig. VI.10b). In these areas no osteoblastic IL-1β mRNA expression was observed (data not shown). Fully formed osteoblastic sites lined with quiescent osteoblast lining cells (fig. VI.11a) showed weak TGFβ1 (fig VI.11b) and little IL-1β mRNA expression (data not shown).

TRANSIENT CYTOKINE EXPRESSION DURING OSTEOPHYTE CLOSURE

With closure of the osteophyte tip, bone formation occurs directly on top of the cartilage. During this phase of bone formation TGFβ1 and IL-1β were observed to be transiently expressed. The start of this bone formation phase associated with osteophyte closure was also observed to occur on top of areas of fibrocartilage cells (fig. VI.12a). At this stage, high levels of TGFβ1 but no IL-1β expression were detected in the fibrocartilage cells (fig. VI.12b and VI.12c). During osteophyte closure osteoblasts were recruited around the periphery of the fibrocartilage cells to initiate bone formation (fig. VI.12d). No TGFβ1 expression was detected at this stage but high IL-1β expression was evident, particularly in the newly recruited osteoblasts (fig. VI.12e and VI.12f).

VI.5 FIGURES

Fig. 4a An area of organised cartilage tissue. There are three zones visible; proliferating and resting chondrocytes (R&P), hypertropic chondrocytes (H), and calcified cartilage (CC).

Fig. 4b Expression of $TGF\beta_1$ mRNA by chondrocytes: note moderate expression in newly formed chondrocytes (single arrow), less expression in hypertropic chondrocytes, and no expression in the calcified cartilage zone (two arrows). Osteoblasts on trabecular bone surfaces (large arrows) express high levels of TGFβ mRNA.

Figure 4c No IL-1β expression detected in a similar region of cartilage tissue.

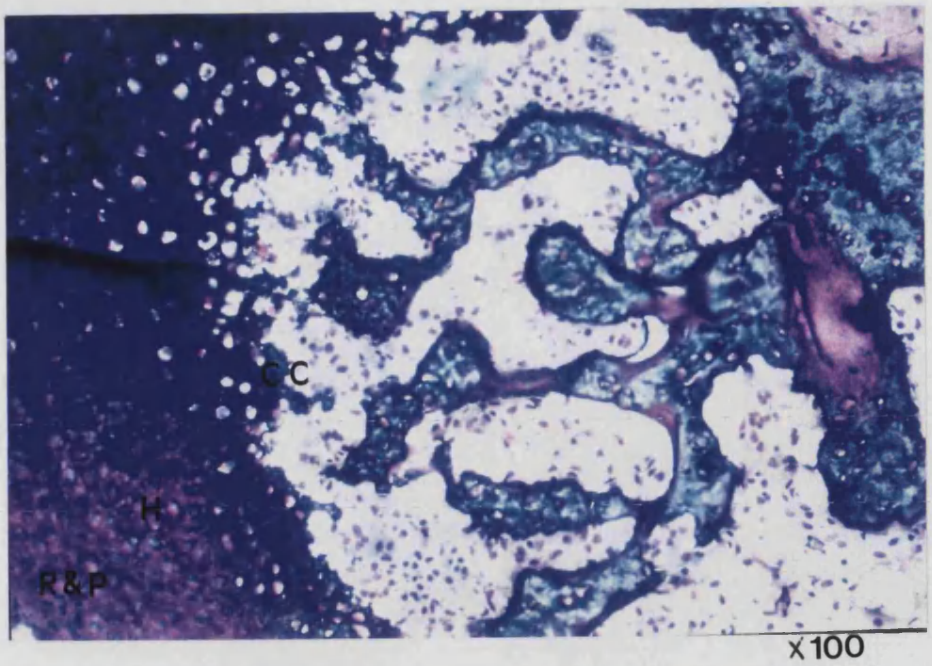


FIG 4a

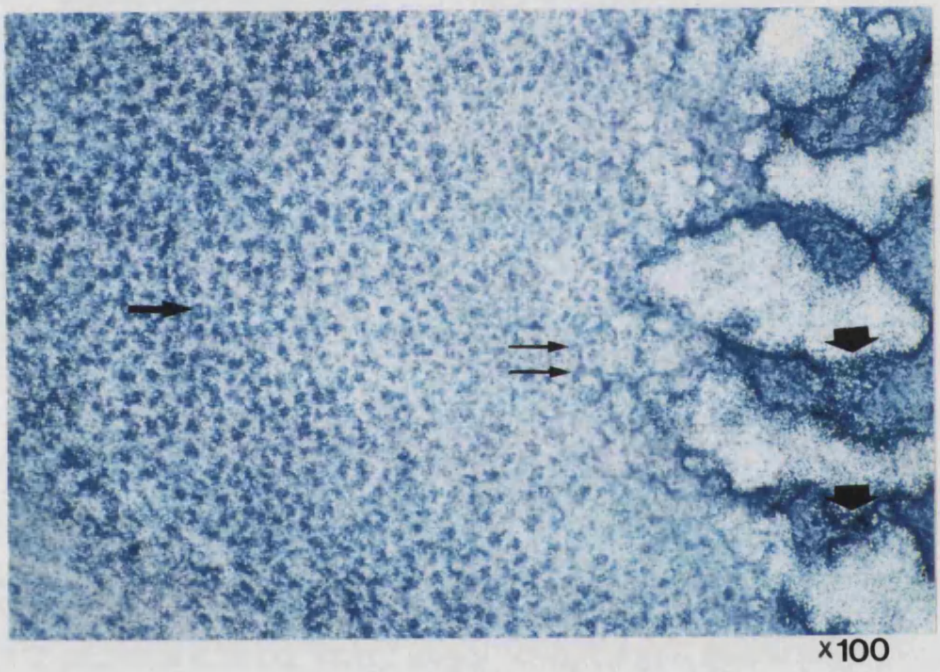


FIG 4b

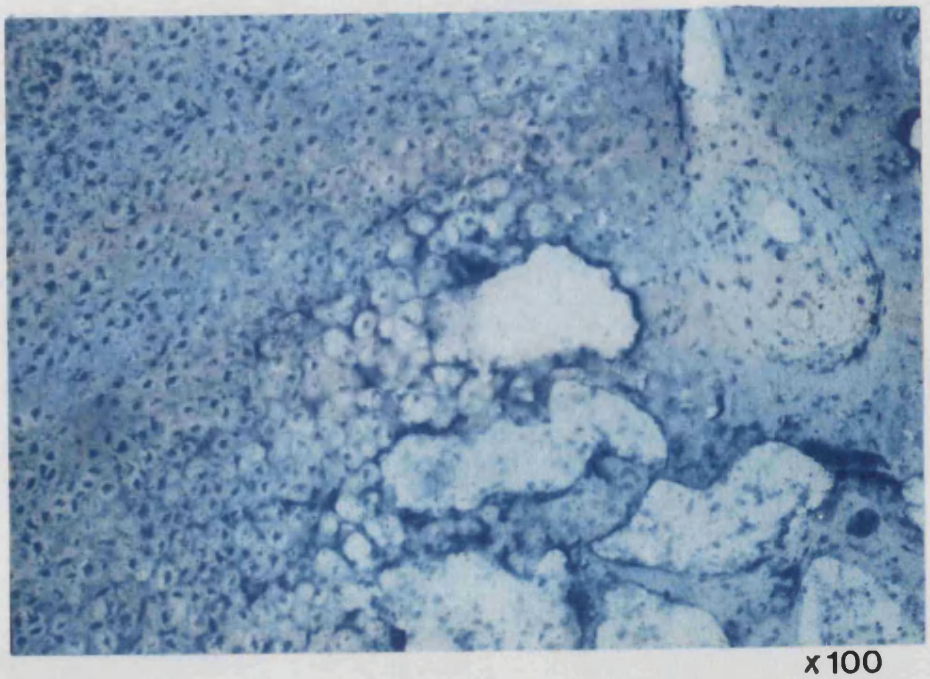
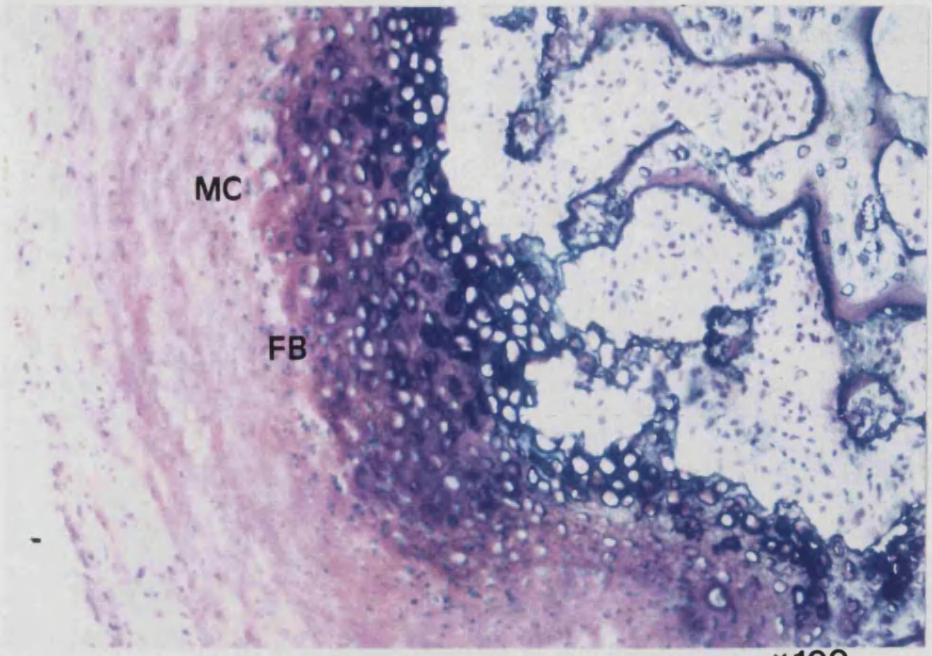


FIG 4c

Fig. 5a Outermost layer of developing osteophyte showing area of fibrocartilage cells (FB) which have differentiated from the surrounding mesenchymal connective tissue (M.C.)

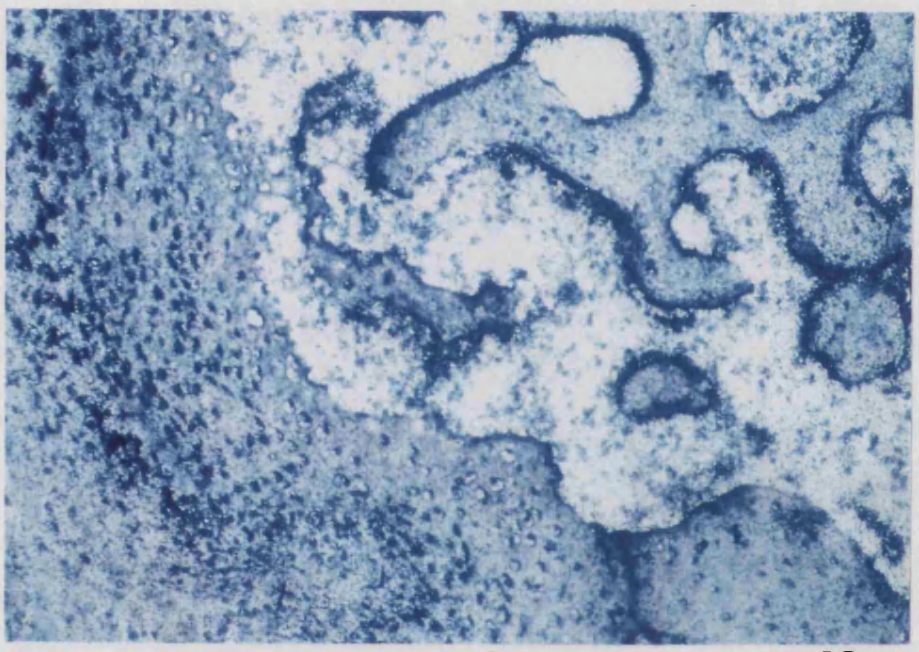
Fig. 5b High expression of $TGF\beta_1$ mRNA in areas of differentiating fibrocartilage cells. Note there is also high $TGF\beta_1$ mRNA expression in osteoblasts on trabecular surfaces.

Fig. 5c Very weak IL-1β mRNA expression in areas of differentiating fibrocartilage, and trabecular bone.



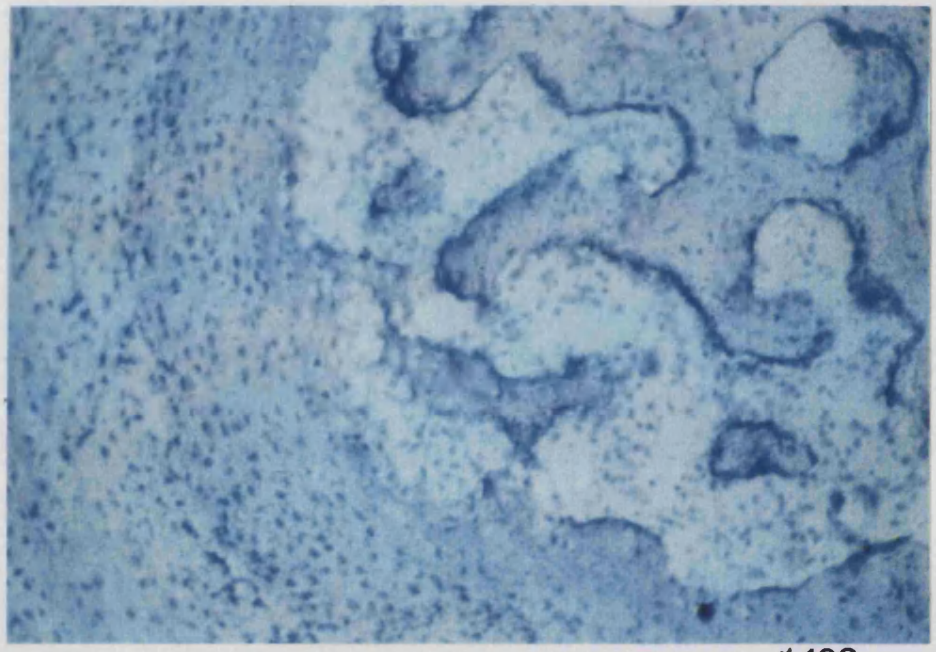
x100

FIG 5a



x100

FIG 5b



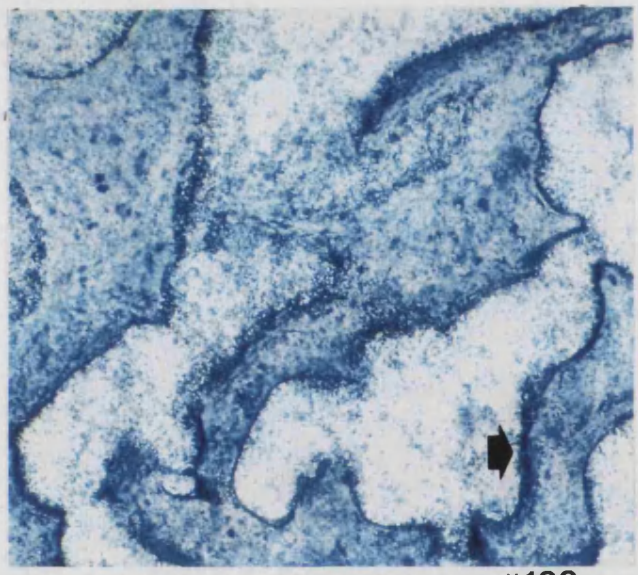
x 100

FIG 5c

Fig. 6a High expression of $TGF\beta_1$ mRNA by osteoblasts apposed to trabeculae of woven bone (large arrow).

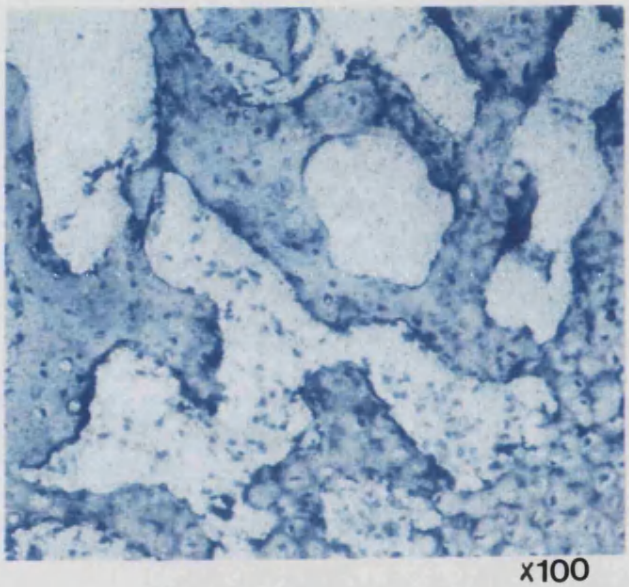
Fig. 6b Negative control showing no binding of sense transcript.

Fig. 6c Very low amounts of IL-1β mRNA detected in occasional osteoblasts (arrow heads).



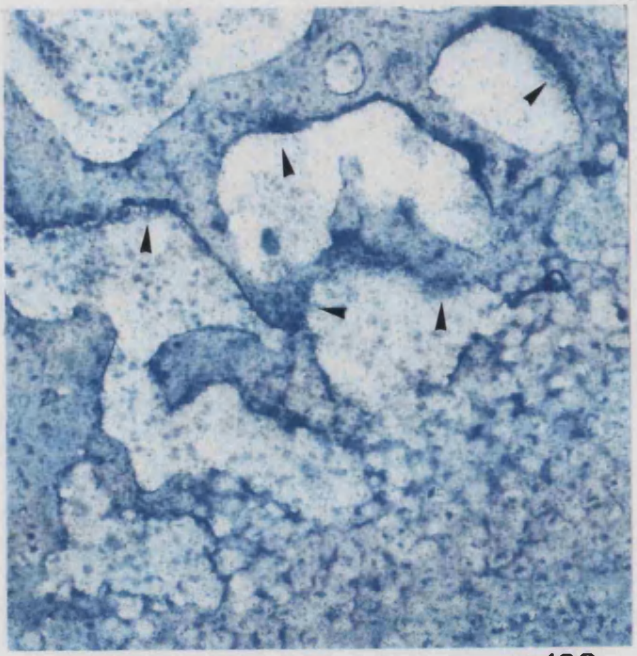
x100

FIG 6a



x100

FIG 6b



x100

FIG 6c

Fig. 7a An area of intramembranous bone formation; islands of woven bone are formed directly by osteoblasts (large arrow) without a cartilage template.

Fig. 7b Osteoblasts express high levels of TGFβ1 mRNA at sites of intramembranous bone formation (large arrows). Note very high expression in clusters of newly differentiated osteoblasts (small arrows).

Fig. 7c Low levels of IL-1β mRNA expression at discrete sites during intramembranous bone formation (small arrows). Lower expression in more flattened osteoblasts (large arrow).

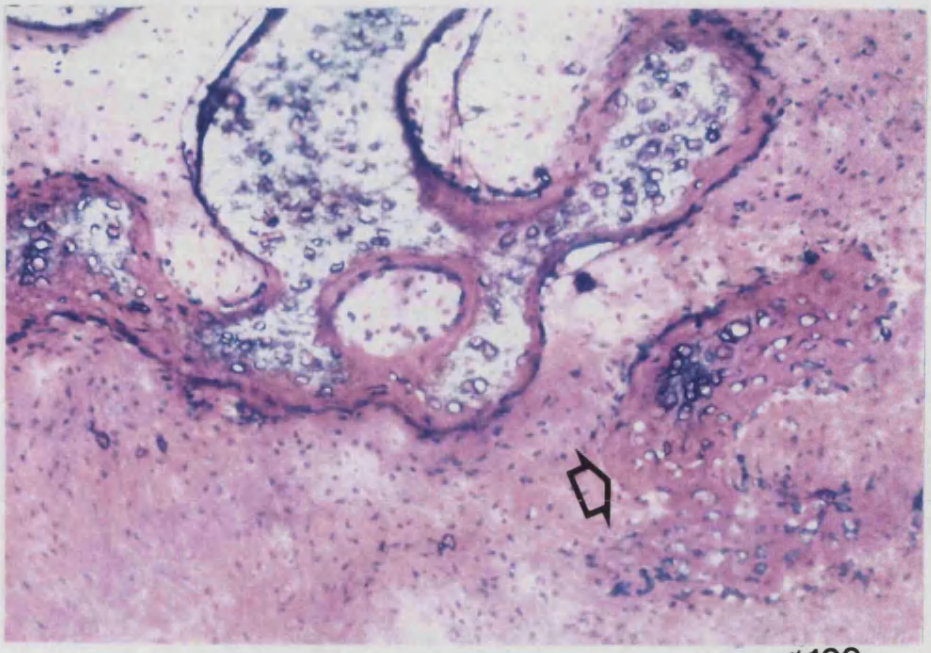


FIG 7a

X100

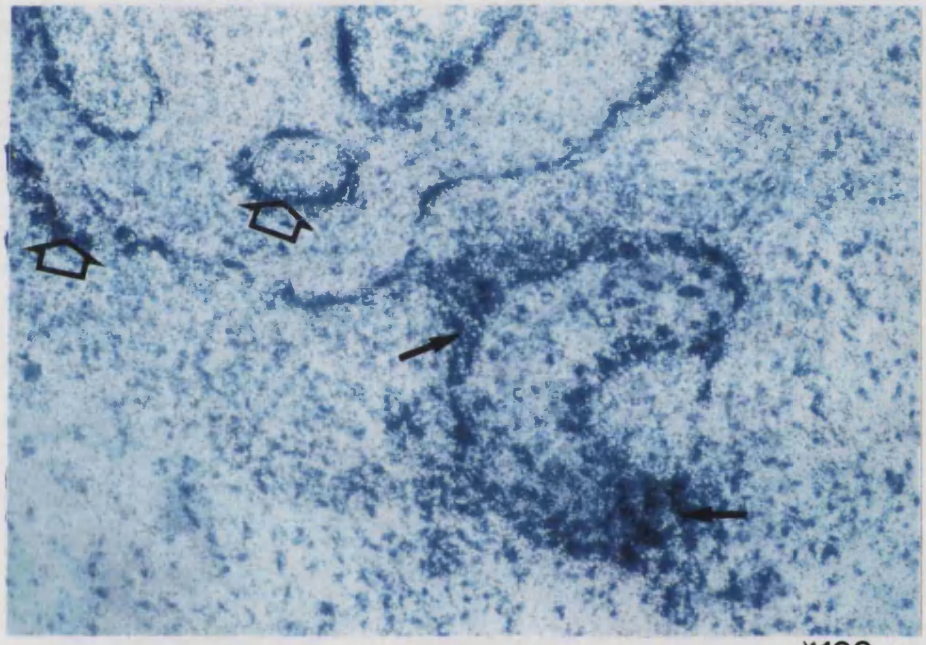


FIG 7b

X100

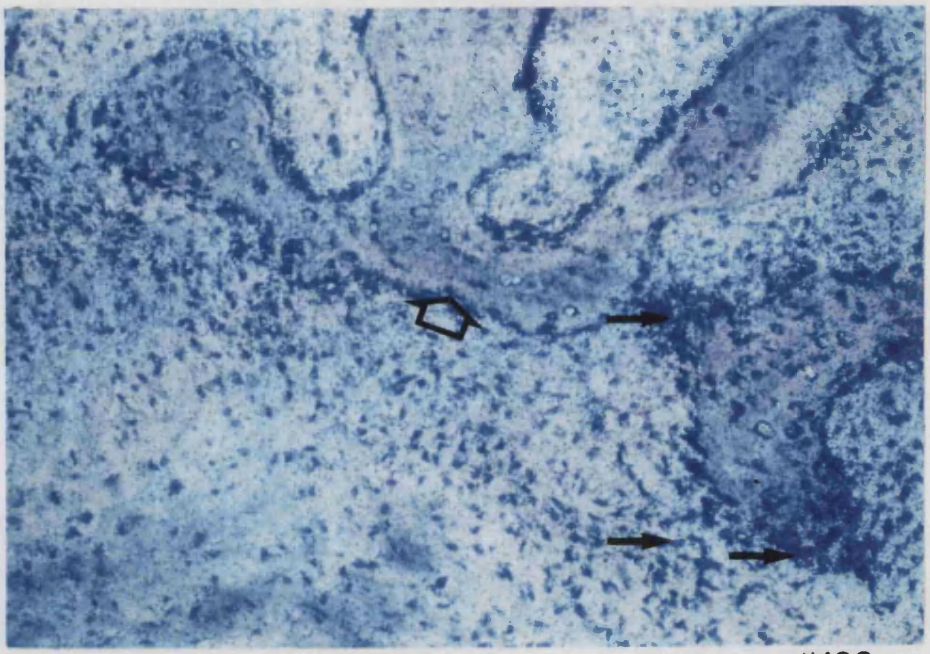


FIG 7c

X100

Fig. 7d Another area of intramembranous bone formation. (Newly differentiated osteoblasts laying down osteoid highlighted by arrows).

Fig. 7e High expression of TGFβ1 by osteoblasts in an area of intramembranous bone formation. Note particularly high expression in newly formed osteoblasts (arrows).

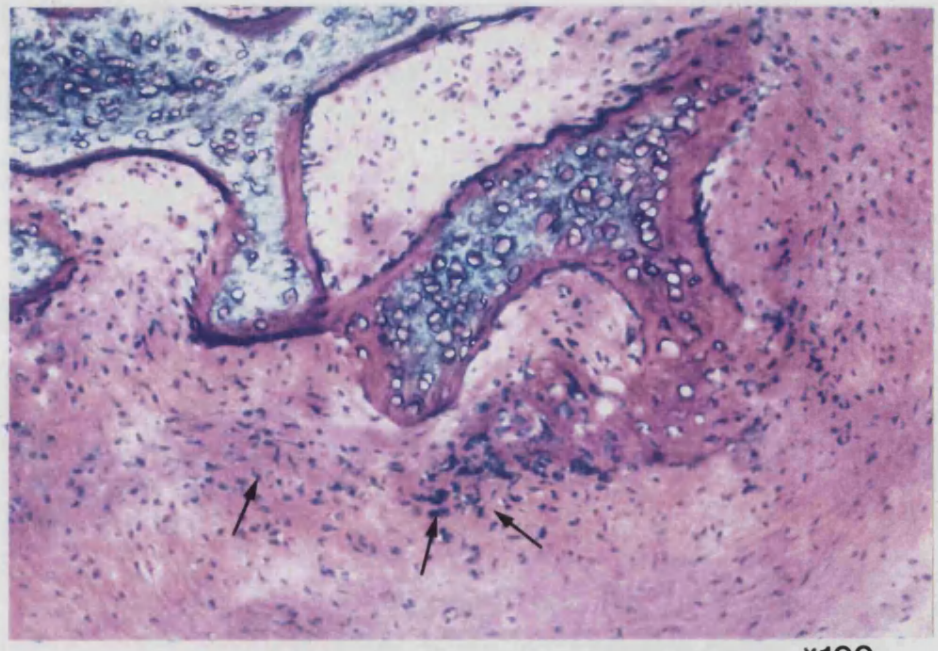


FIG 7d

x100

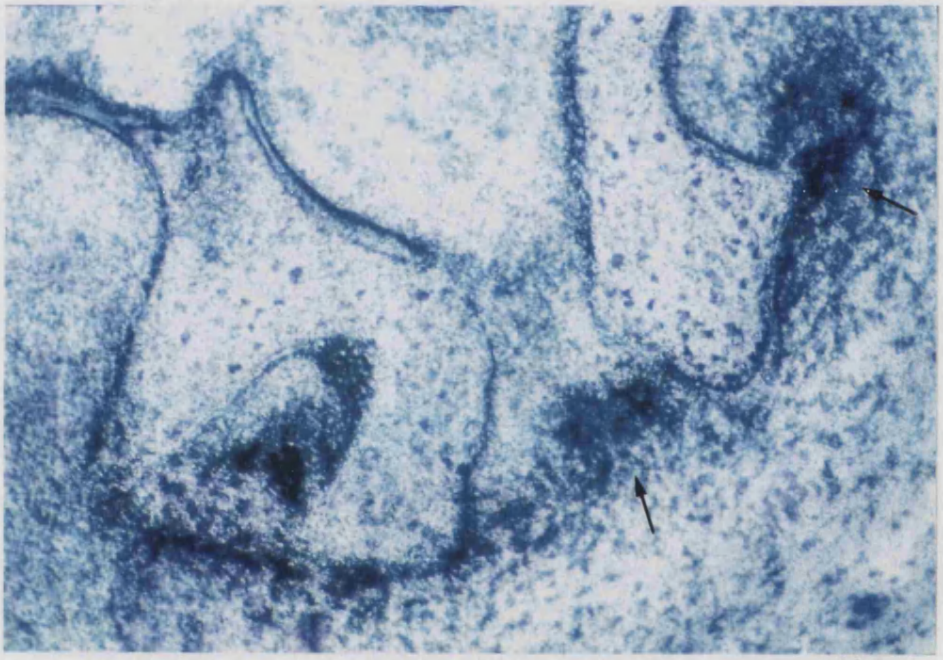


FIG 7e

x100

Fig 8a An area of intramembranous bone formation; "bundles" of osteoblasts (arrows) can be seen to be laying down osteoid.

Fig. 8b High IL-1β expression by "bundles" of osteoblasts laying down osteoid at a site of intramembranous bone formation.

Fig. 8c Negative control showing lack of binding of sense transcript.

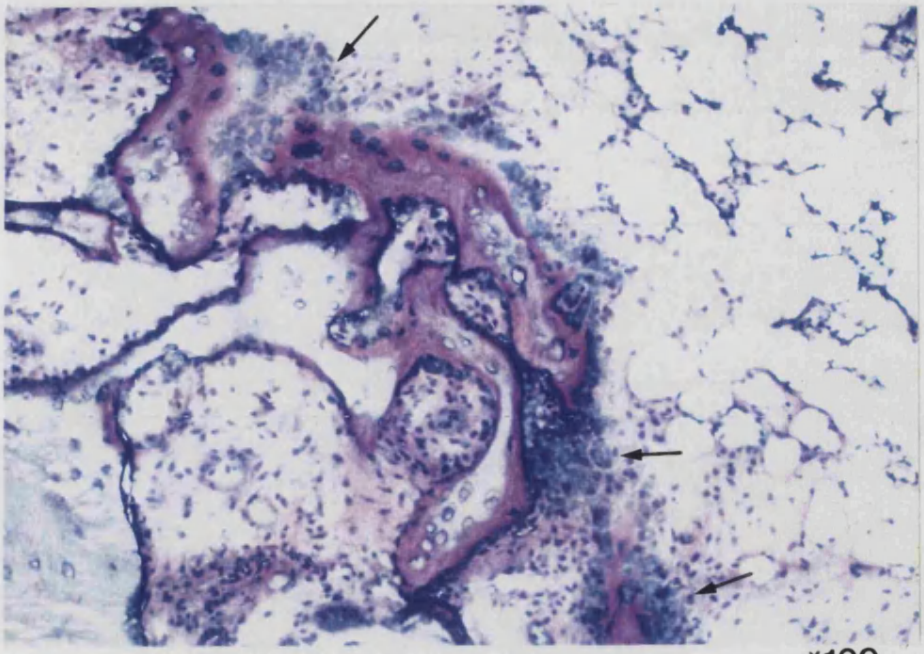


FIG 8a

x100

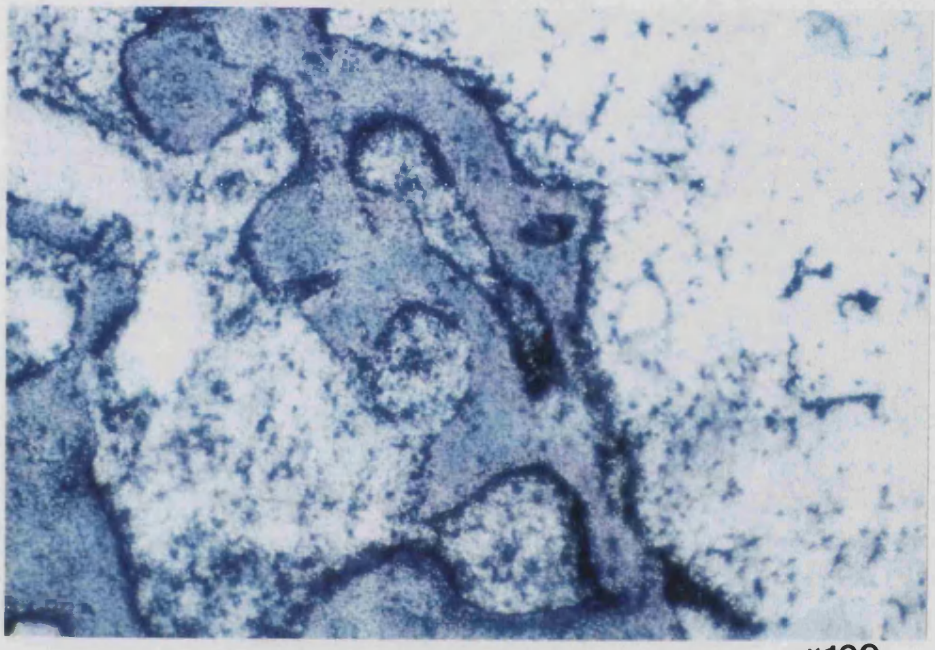


FIG 8b

x100

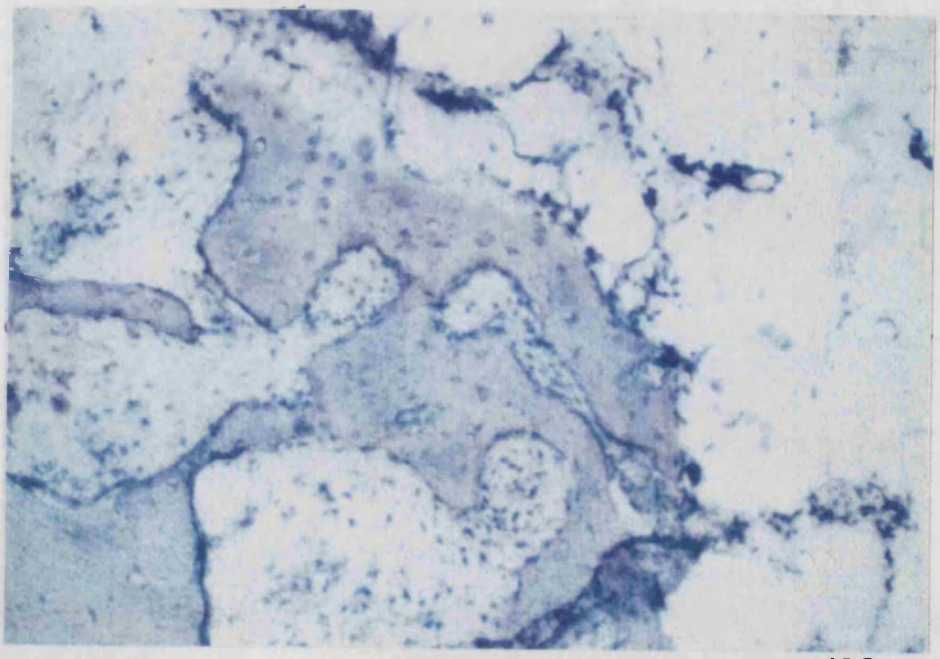
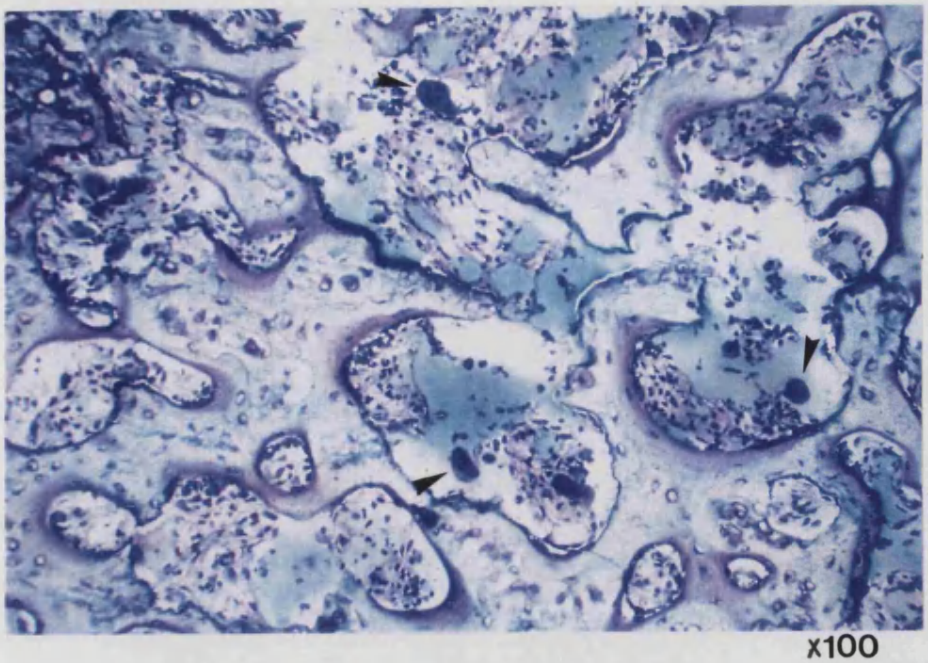


FIG 8c

x100

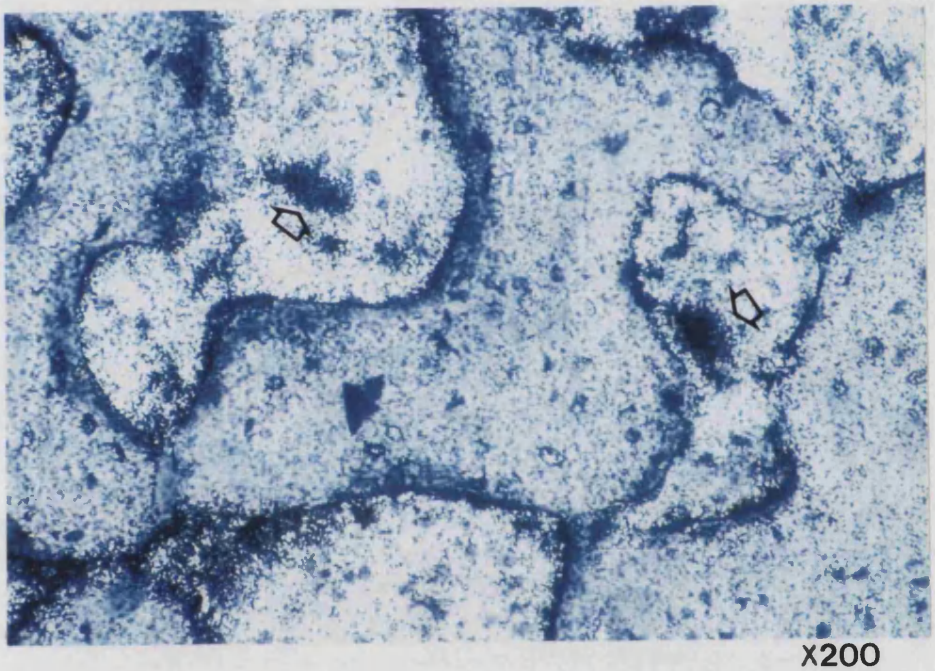
Fig. 9a Extensive bone remodelling by osteoclasts. The highly cellular marrow between trabeculae contains mononuclear stromal cells and migrating osteoclasts (arrow heads).

Fig. 9b An area of remodelling bone; large multinuclear cells resembling osteoclasts (large arrows) express high levels of TGFβ1 mRNA.



x100

FIG9a



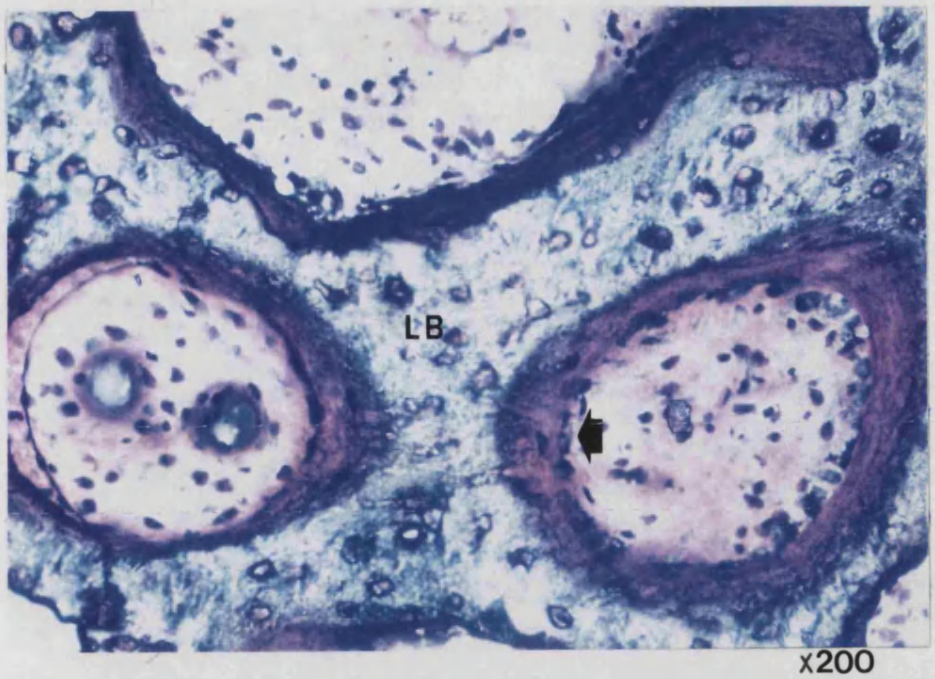
x200

FIG9b

Fig. 10a An area of lamellar bone formation; seams of newly formed osteoid are evident (arrow) apposed to lamellar bone (LB).

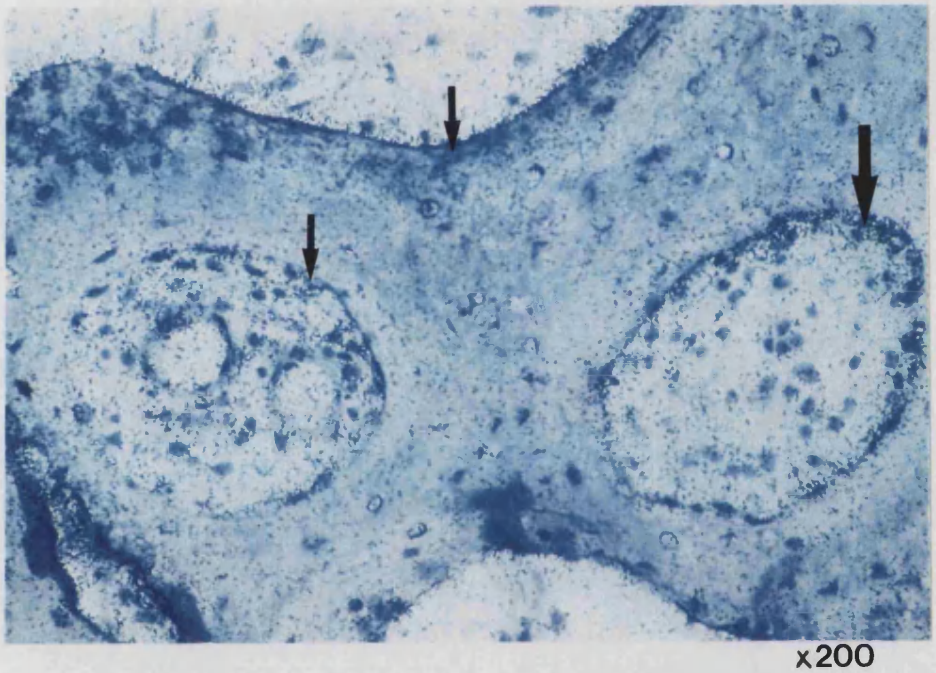
Fig. 10b Osteoblasts express low levels of TGFβ1 mRNA during lamellar bone formation. Higher levels were detected in plump cuboidal osteoblasts (large arrow), and less expression in those with a flattened morphology (small arrow).

Fig. 10c No expression of IL-1β during lamellar bone formation.



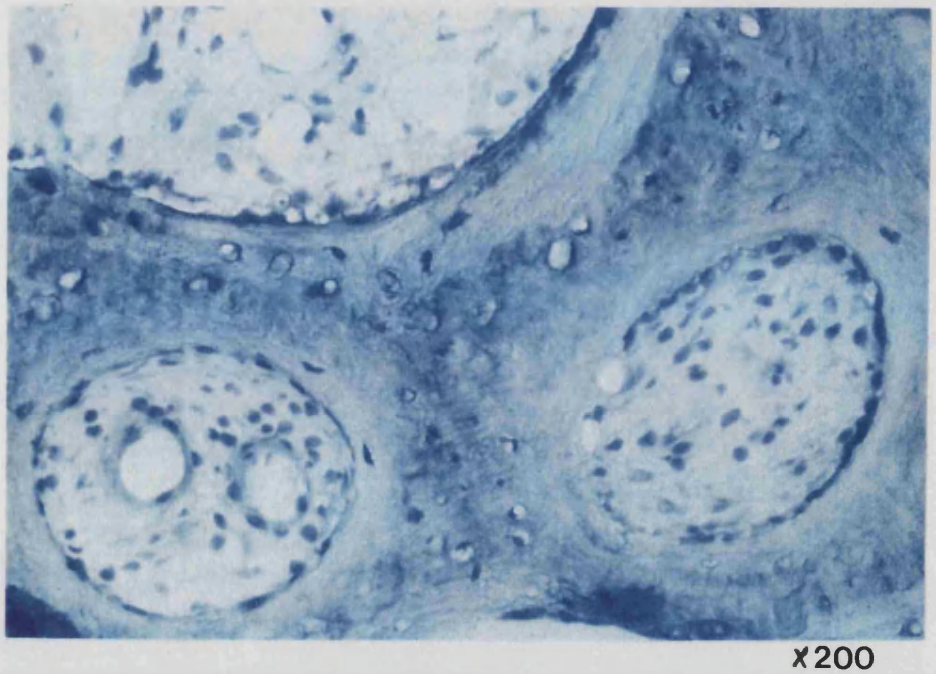
x200

FIG10a



x200

FIG10b



x200

FIG10c

Fig. 11a An area of residual woven bone formation.

Fig 11b. Fully formed osteoblastic sites show weak expression of TGFβ1 mRNA (large arrows); quiescent lining cells show no expression (small arrows).

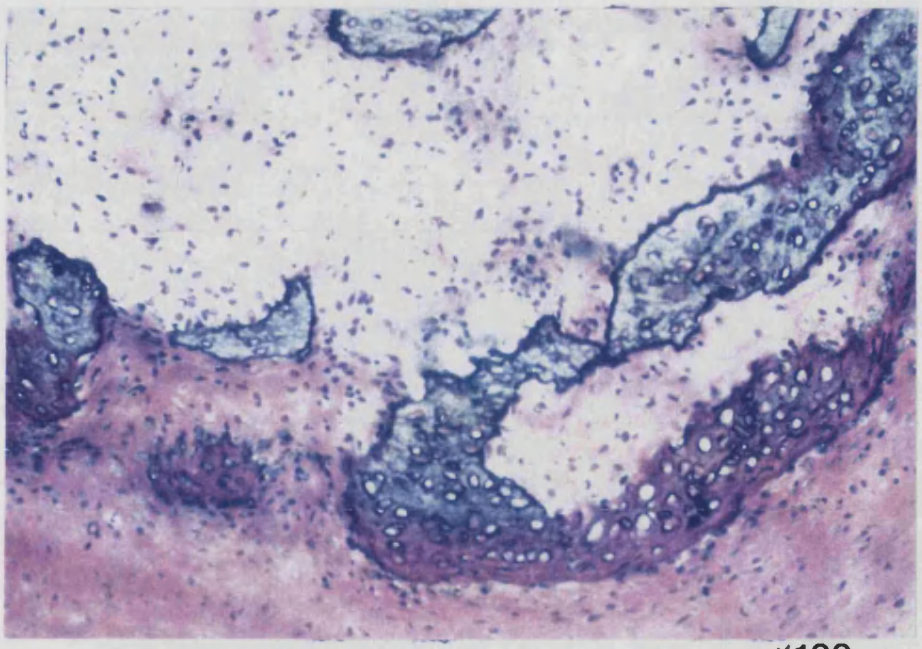


FIG11a

x100

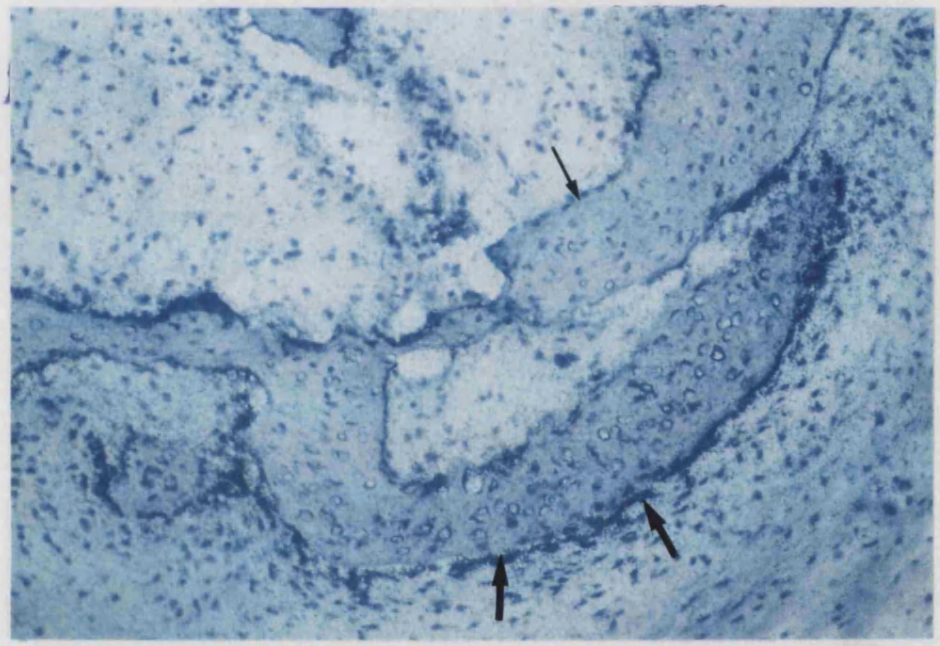


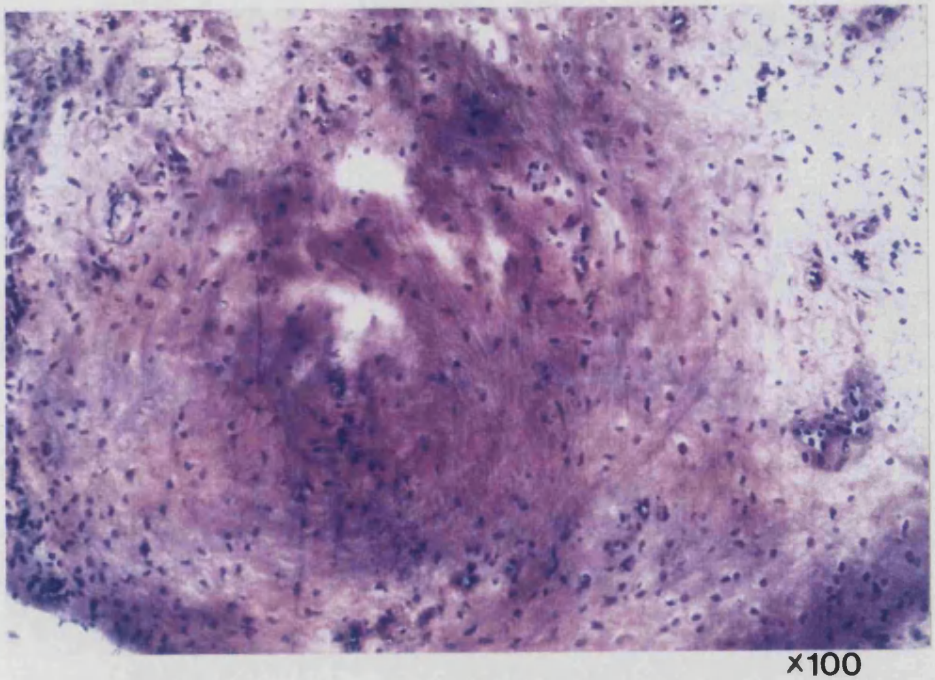
FIG11b

x100

Fig. 12a Tip of the osteophyte showing fibrocartilage cells surrounded by a cartilagenous matrix.

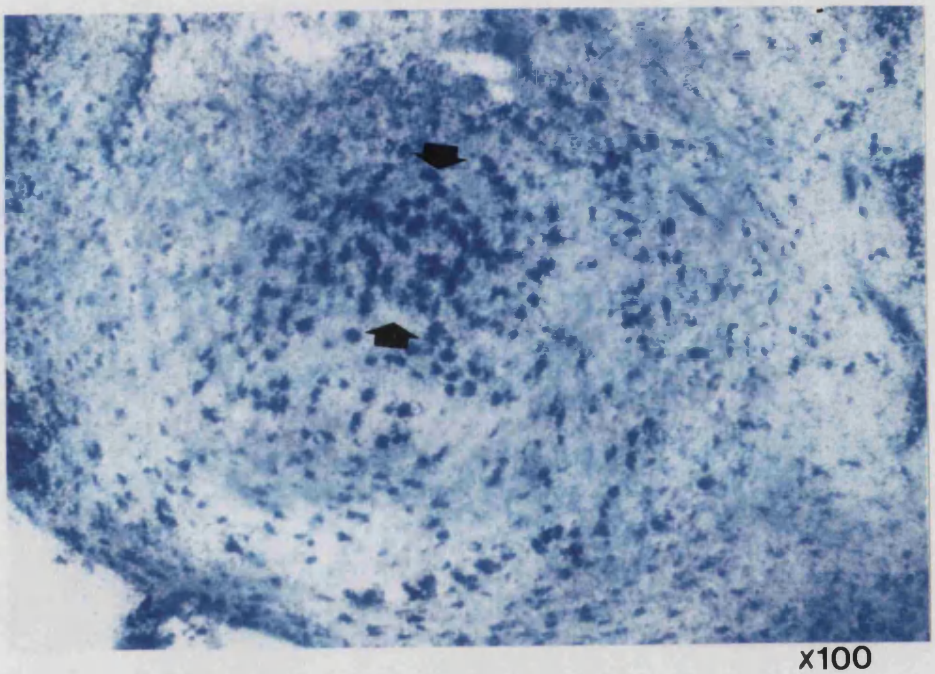
Fig. 12b Moderate expression of TGFβ1 mRNA in fibrocartilage cells (arrows).

Fig. 12c No expression of IL-1β mRNA at this stage of osteophyte closure.



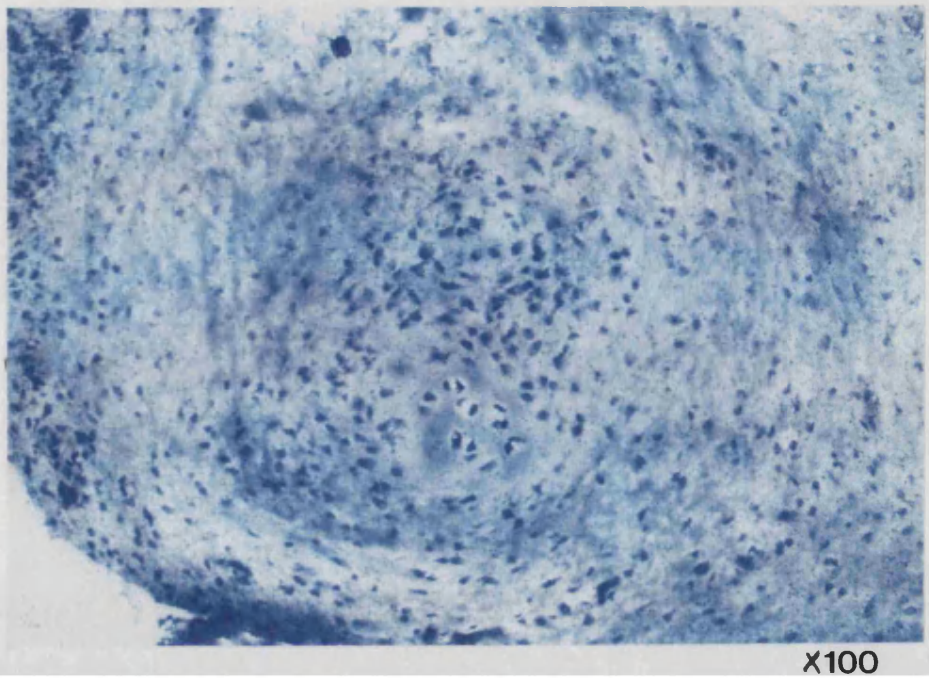
x100

FIG12a



x100

FIG12b



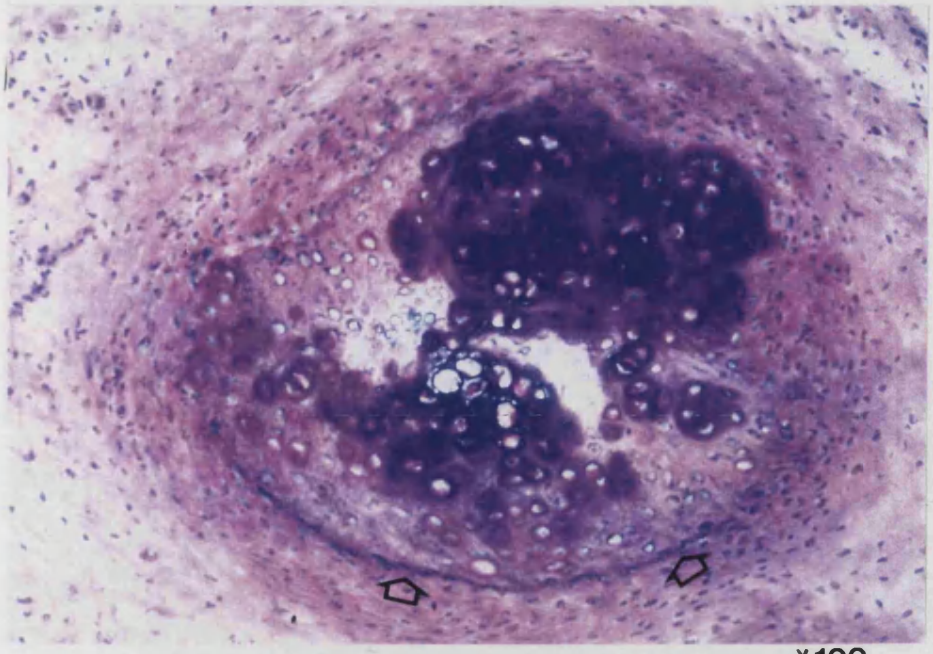
x100

FIG12c

Fig. 12d A more mature stage of osteophyte closure. Clusters of fibrocartilage cells can be seen in the centre surrounded by a layer of migrating osteoblasts (arrrows).

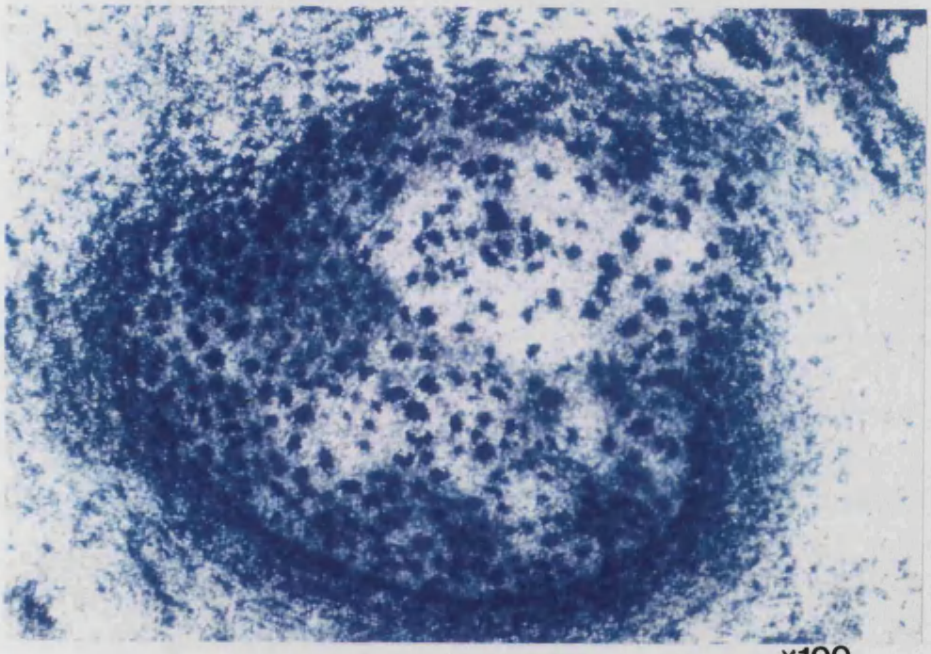
Fig. 12e High IL-1β expression evident particularly in newly recruited osteoblasts.

Fig. 12f No TGFβ1 expression detected at this stage of osteophyte closure.



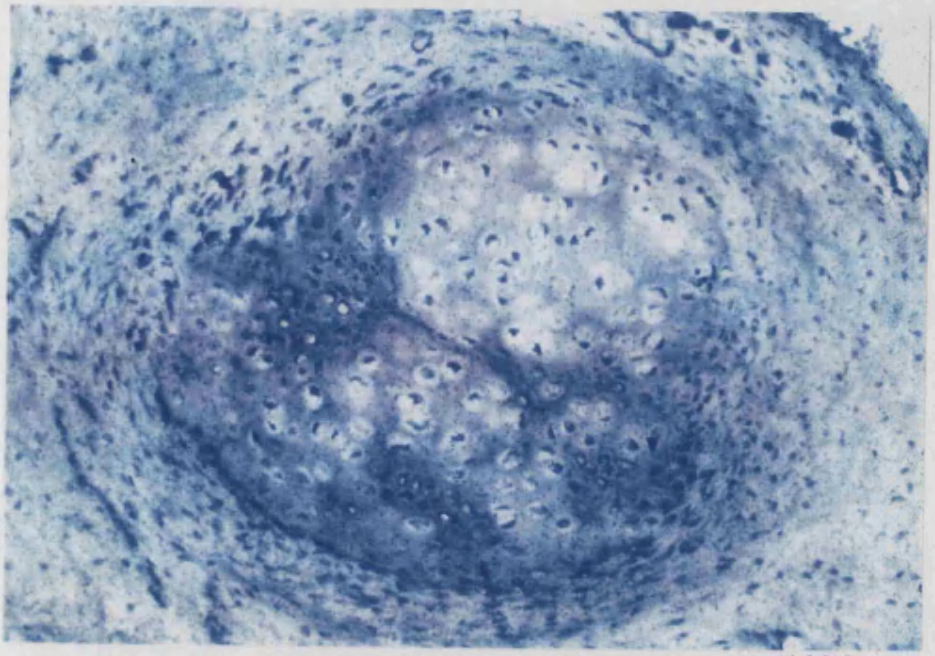
X100

FIG12d



X100

FIG12e



X100

FIG12f

Table VI.1. Summary of TGFβ, and IL-1β expression within the developing osteophyte.

CELL TYPE	CYTOKINE EXPRESSION	
	TGFβ	IL-1β
Connective tissue.		
Differentiating fibrocartilage.	+++	+/-
Fibrocartilage.	+	-
Forming chondrocyte clusters.	+	*
Mature chondrocytes.	+	*
Hypertrophic chondrocytes.	+	*
Calcified cartilage (chondrocytes).	-	-
Osteoblasts.		
(a) Differentiating osteoblasts (IMB).	+++	*
(b) Intramembranous bone formation.	++	*
(c) Quiescent IMB surfaces.	+	-
(d) Apposed CC (EO).	+/-	-
Trab: (woven) / "active obs" (osteoid).	+++	*
Lining cells.	+/-	-
Trab: (woven - lamellar) / "active obs".	++	*
Lining cells.	+	-
Osteoclasts.	**	-

Key

-	NO EXPRESSION
+/-	WEAK/NO EXPRESSION
*	TRANSIENT EXPRESSION; generally negative.
**	VERY OCCASSIONAL OSTEOCLAST (++)
EO	ENDOCHONDRAL OSSIFICATION.
IMB	INTRAMEMBRANOUS OSSIFICATION.
CC	CALCIFIED CARTILAGE

VI.5 DISCUSSION

This study confirms the expression of the cytokines TGFβ and IL-1β in human bone. These findings are very important, and are the most compelling evidence that local cytokine expression is involved in the control of human bone remodelling. There has been a large body of evidence from *in vitro* experiments suggesting that the cytokines TGFβ1 and IL-1β are important osteotropic factors. However until the completion of this study it was possible that the *in vitro* expression of these cytokines, could be as a result of culture conditions.

Many different cell types were shown to be capable of expressing mRNAs for these factors as summarised in table VI.1. The expression of the cytokines was shown to occur at specific stages of development. Each cytokine was not expressed by a certain cell type continuously, but rather transient expression was observed. Factors such as the differentiation state of the cell and the type of bone remodelling seemed to modulate the expression of each cytokine.

The expression profile observed for TGFβ1 confirmed the data from many *in vitro* experiments, where osteoblasts from several different species have been observed to express TGFβ (Gehron Robey et al.1987; Pfeilschifter and Mundy, 1987). The most interesting aspect of TGFβ1 expression, was the way it seemed to be dependent on the differentiation stage of each cell. This phenomenon was seen in both cartilage cells and osteoblasts, where the more immature stages expressed much higher levels of TGFβ1 mRNA. It is possible that TGFβ itself is acting to promote the maturation of the cell, or that it is inducing the expression of another maturation factor. There is evidence to support this hypothesis since TGFβ has been observed to promote chondrocyte maturation *in vitro* (Morales and Roberts, 1988).

It is possible that TGFβ and IL-1β expression by osteoblasts might modulate local bone remodelling by acting as autocrine growth factors. It was evident that the expression of both the cytokines was particularly high during intramembranous bone formation.

During this stage large bundles of osteoblasts were observed at sites of bone formation, presumably they must result from active osteoblast proliferation. Both TGFβ and IL-1 have been shown to stimulate the proliferation of human osteoblasts *in vitro* (D. Rickard,personal communication). Additionally, work reported in Chapters III and IV has shown that both factors autoinduce the expression of their own mRNAs, and human osteoblasts express functional TGFβ1 and IL-1β receptors. Therefore it is suggested that during stages of bone formation where large numbers of osteoblasts are required to synthesise osteoid, both these factors are concurrently expressed to stimulate osteoblast proliferation. In addition, this proliferative signal may be amplified by the autoinduction of both of the cytokine mRNAs.

It is possible that IL-1β expression was transient in these circumstances to avoid matrix degradation. Since, in addition to stimulating osteoblast proliferation, IL-1 inhibits the synthesis of many bone matrix components including type I collagen (Canalis, 1986). Additionally IL-1 is reported to promote the synthesis of metalloproteinases, that degrade extracellular matrix, by chondrocytes (Schnyder et al. 1987). The transient expression profile and short mRNA half-life of IL-1β, may allow it to stimulate osteoblast proliferation, whilst avoiding inhibiting osteoid synthesis, or promoting matrix breakdown.

In contrast, TGFβ has been reported to stimulate the expression of certain bone matrix proteins including fibronectin, type I collagen (Ignotz and Massague, 1986; Strong et al. 1991) and the metalloproteinase inhibitor TIMP (Meikle et al.1991) by osteoblasts. This might explain why TGFβ expression is more sustained and expressed highly by osteoblasts actively laying down bone matrix.

High TGFβ expression was also seen when large numbers of osteoblasts were required to synthesize bone during osteophyte closure. In addition to stimulating the proliferation of osteoblasts, TGFβ has been shown to act as a chemotactic factor for these cells *in vitro* (Pfeilschifter et al.1990). During osteophyte closure TGFβ was observed to be transiently expressed at a time when there was active osteoblast

recruitment to the site of bone formation. Therefore it is suggested that this factor is transiently expressed by fibrocartilage cells to facilitate this migration.

The fact that TGFβ expression was seen in most sites of intramembranous bone formation, whilst IL-1β expression was transient and rarely observed is very interesting. It is important to realise that the expression of only two members of a whole cytokine network were studied in these experiments, and that other factors are also probably modulating the expression of these cytokines. It is probable that the rare cases of IL-1β expression during intramembranous bone formation are due to some unknown locally produced factor.

High levels of TGFβ expression were detected in large multinuclear cells during lamellar bone remodelling. It is tempting to speculate that these cells were osteoclasts, as many were observed in adjacent histology sections during this stage of remodelling. There has been one previous report that osteoclasts are capable of expressing TGFβ in human fetal bone (Sandberg et al.1988). However these findings would directly contravene the whole "coupling factor" theory, where TGFβ is thought to couple resorption to formation during bone remodelling. The critical prerequisite of this theory is that TGFβ is synthesized by osteoblasts in a latent form and deposited in the bone matrix. Subsequently it is thought to be liberated from the bone matrix and activated during further rounds of remodelling by osteoclasts. If osteoclasts could synthesize their own TGFβ the theory would have to be substantially modified.

This highlights a property of TGFβ which is important to consider when interpreting this entire *in situ* study, namely that it is assumed that TGFβ is in a latent form. Therefore if this doctrine is accepted no TGFβ activity can occur until this factor is activated by acidification or proteolysis (Oreffo et al.1989). The fact that TGFβ is expressed during distinct stages of osteophyte development suggests that it is not just secreted in a latent form, but rather that it may be activated in at its site of synthesis, by an as yet uncharacterized mechanism. There is some evidence to support this theory since it has been reported that chick chondrocytes produce active $TGF\beta_3$ in certain

circumstances (Loverige and Roberts,personal communication).

High transient expression of IL-1β mRNA was also detected during osteophyte closure, particularly by the layer of osteoblasts surrounding the central fibrocartilage cells. By examining subsequent sections during osteophyte closure it was observed that as TGFβ1 expression was "switched off", IL-1β expression was "switched on". This differential expression of the two cytokines at distinct stages of osteophyte development, further supports the hypothesis that the local production of these two factors is important in the regulation of bone and cartilage remodelling.

It is possible that the expression of these two cytokines could be irrelevant to bone remodelling. Other cytokines might be the critical factors controlling the remodelling, the TGFβ1 and IL-1β expression might just be coincidental. This is thought unlikely for two reasons. Firstly, there has been such a large amount of data documenting the osteotropic effects of TGFβ1 and IL-1β *in vitro*, and secondly, if expression was coincidental it is very unlikely to occur at distinct stages of differentiation or osteophyte development.

There are many problems and pitfalls with the experimental design and interpretation of *in situ* experiments. The main problem is that a section just highlights the expression of a factor at a certain time point. If the expression of the factor is "switched" on and off quickly it is possible to miss the expression. This problem is particularly relevant in the case of cytokines (including IL-1β) which are transiently expressed and have very short messenger RNA half-lives. It was expected that large amounts of IL-1β expression would be detected in osteoblasts during active woven bone remodelling, since IL-1 has been shown to be a potent bone resorptive agent *in vitro* (Gowen et al.1983). However very little expression was detected in these stages, and it is possible that due to the aforementioned reasons IL-1β went undetected.

The other possible criticism of this study was the choice of tissue. As mentioned previously, osteophyte tissue was chosen because it contains an abundance of bone cell types within areas of very active bone remodelling, that are not easily found in normal

adult human bone. It is possible however that as this bone forms as part of a disease process, the remodelling observed may not reflect true bone remodelling. From an extensive characterisation of this tissue (Dodds and Gowen, 1992), it is thought that whilst the kinetics of bone remodelling are accelerated, the actual phases of bone remodelling that occur are very similar to normal human bone remodelling. For example the process of intramembranous bone formation that was observed, where osteoblasts differentiate from the mesenchyme and lay down areas of osteoid, has been previously described to occur in normal bone tissue (Baron, 1991).

The main problem of investigating human bone remodelling is that the experimental design cannot be manipulated to investigate the affect of other osteotropic factors. Therefore it is necessary to compliment these studies with *in vitro* experiments of human bone cells. It would be very advantageous to monitor the expression of the cytokines at the protein level during the various stages of bone remodelling by immunlocalization techniques, since some studies have documented mRNA expression without translation into protein (Ikejina et al.1987). Unfortunately this was not possible in this tissue for technical reasons.

Further studies on normal human bone would lead to a greater understanding of human bone remodelling. It is possible to obtain iliac crest biopsies from patients with metabolic bone disorders or those on clinical drug trials. In certain cases such patients are injected with compounds (for example tetracyclin) which are deposited in the bone matrix during phases of bone formation. These compounds are fluorescent when viewed under u.v. light, and thus phases of bone formation and the time between such formation phases can be monitored. If *in situ* hybridization of cytokine probes was carried out in serial sections of these bone biopsies, it might be possible to determine at which stages of the bone remodelling cycle each cytokine was expressed. Preliminary pilot studies for this type of work have been carried out. Unfortunately the iliac crest tissue is very difficult to work with and much tissue is lost during the long *in situ* hybridization technique.

It is hoped that with the advent of more sophisticated techniques, such a study could be undertaken in the future.

Chapter VII: Osteopontin expression in the developing osteophyte as detected by *in situ* hybridization.

VII.1 ABSTRACT

Over recent years several noncollagenous matrix proteins of bone have been isolated and characterised. One such protein osteopontin, has been shown to be synthesized by osteoblasts and deposited in the bone matrix where it is thought to bind to hydroxyapatite.

In this study, *in situ* hybridization techniques have been used to investigate the expression of osteopontin mRNA by bone cells in a variety of bone environments. Cryostat sections of human osteophyte and osteoclastoma tissue were hybridized with an antisense RNA probe for osteopontin.

There was a very distinct pattern of osteopontin mRNA expression in these tissues. Osteoblasts actively secreting osteoid adjacent to the bone surface expressed high levels of osteopontin mRNA. Osteopontin expression was also detected in low levels in chondrocytes and in very high levels in fibrocartilage cells at the periphery of the osteophyte.

However the most striking feature of osteopontin expression was the high levels of osteopontin mRNA detected in osteoclasts. Osteoclasts in resorption lacuna and those removed from the resorption sites both expressed osteopontin mRNA. In addition, the whole population of osteoclasts in the osteoclastoma tissue expressed high levels of osteopontin mRNA. Populations of mononuclear cells in resorption lacunae were also shown to express high levels of osteopontin mRNA.

VII.2 INTRODUCTION

Osteopontin was first isolated from mineralised bone matrix by Franzen et al. (1985). It was originally named bone sialoprotein I as it was found to contain many sialic acid moieties (Franzen and Heinegard, 1985). Osteopontin has been shown to be a minor matrix component of bovine bone; the amount detected in different bone matrices was species dependent, but was on average approximately 2% of the non collagenous proteins (Franzen and Heinegard, 1985). In addition to containing sialic acid moieties osteopontin has been shown to be heavily glycosylated as a result of post translational modifications. This often gives rise to different molecular weight species due to varying glycosylation patterns (Zhang et al.1990). For this reason osteopontin migrates anomalously on SDS PAGE, and has an estimated molecular weight between 45kDa - 75kDa. Sedimentation analysis seems to give a more reproducible estimate of 44kDa (Franzen and Heinegard, 1985). Osteopontin binds tightly to hydroxyapatite in bone presumably via an acid rich stretch of nine aspartic acid residues. It appears to form an integral part of the mineralised matrix since it can only be extracted under denaturing conditions (Fisher et al.1987).

Osteoblasts obtained from many species have been shown to be capable of synthesizing osteopontin *in vitro*, including osteoblasts isolated from fetal rat calvariae and the rat osteoblast cell line ROS 17/2.8 (Prince et al.1987). Synthesis of osteopontin by normal human bone cells has not been documented, but it has been suggested that it is not a major secretory product of these cells (Gehron Robey, 1989). Osteopontin is also synthesized by chondrocytes, where it's expression seems to be restricted to areas of active cartilage remodelling (Castagnola et al.1991).

Osteopontin has been immunolocalized in osteoblasts, osteocytes and preosteoblasts in the woven bone of neonatal rats (Mark et al.1987). In the same study the intracellular localization of the osteopontin was shown to be the Golgi apparatus, presumably since that was the site for the extensive glycosylation of the protein. *In situ* hybridization

studies of developing mouse limb buds suggested that osteopontin mRNA was present in fibroblast-like cells internal to the periosteum and it's associated matrix (Nomura et al.1988). Analysis of the temporal expression of osteopontin during embryogenesis revealed that it was expressed 14 days postcoitum in calvariae in a similar manner to osteonectin (Nomura et al.1988). Electron microscopy studies have localized osteopontin to electron dense regions of mineralised matrix (Reinholt et al.1990), particularly in regions of the bone surface where osteoclasts are anchored. Therefore it may act as a means of attachment for these cells to the bone surface (Reinholt et al.1990).

For a long time it was assumed that osteopontin was a specific bone matrix protein. However a study using Northern blot analysis by Yoon et al (1987) showed this was not the case. Osteopontin RNA transcripts were detected in high levels in bone and kidney. However recent work suggests that the kidney form may be genetically distinct (Gotoh et al.1991). Lower levels of osteopontin expression were also detected in skin, muscle, cartilage, heart, intestine, liver, spleen and testes. Subsequent immunolocalization studies demonstrated that neuronal cells in the brain and inner ear express both osteopontin mRNA and protein (Nomura et al.1988).

Even though osteopontin is not a bone specific protein, its expression is modulated by osteotropic factors. The synthesis of osteopontin by osteoblasts is stimulated by 1,25 D_3 (Prince and Butler, 1987) and TGFβ (Noda and Rodan, 1989) and inhibited by parathyroid hormone (Noda and Rodan, 1989) and dexamethasone (Yoon et al.1987). Further evidence that osteopontin may be important in bone metabolism arises from an analysis of the osteopontin promoter. In addition to a vitamin D responsive element a sequence which confers oestrogen responsiveness has also been discovered (Craig and Denhardt, 1991).

Sequence analysis of the osteopontin gene reveals that it contains an RGD tripeptide sequence and therefore may interact with integrin receptors (Kiefer et al.1989). It has been postulated to be a ligand for the vitronectin ($\alpha_v\beta_3$) receptor (Reinholt et al.1990). Osteopontin may also be important in cell growth as it can be shown to promote

collagen independent cell spreading and attachment of ROS17.28 cells and normal human osteoblasts in culture (Gehron Robey et al.1989).

Despite extensive studies on osteopontin in several species its precise function in bone formation and remodelling remains to be fully elucidated. For this reason a study of the expression of osteopontin in human bone was undertaken. *In situ* hybridization of RNA probes for osteopontin to cryostat sections of human bone and osteoclastoma tissue was used to investigate its expression at different stages of bone remodelling. Human osteophyte tissue was used for two reasons: the tissue contains areas of active bone formation and resorption, and therefore is rich in the various cell types of bone. Secondly, this tissue has been extensively characterised (Dodds and Gowen 1992) Osteoclastoma tissue was used as a rich source of osteoclasts and stromal cells that were not in a bone environment. Serial bone and osteclastoma sections were stained with a Wrights stain to visualize the histology of the tissue. Two enzymes that are expressed highly by osteoblasts and osteoclasts, alkaline phosphatase and tartrate resistant acid phosphatase (TRAP) were visualized by cytochemical methods to distinguish osteoblasts and osteoclasts in the sections. Using this approach the aim of the study was to define which cell types were capable of expressing osteopontin mRNA and to characterise the bone microenvironments in which the transcription of this protein was initiated.

VII.3 MATERIALS AND METHODS

The tissues were prepared in exactly the same manner as described in chapter VI.

In situ hybridization.

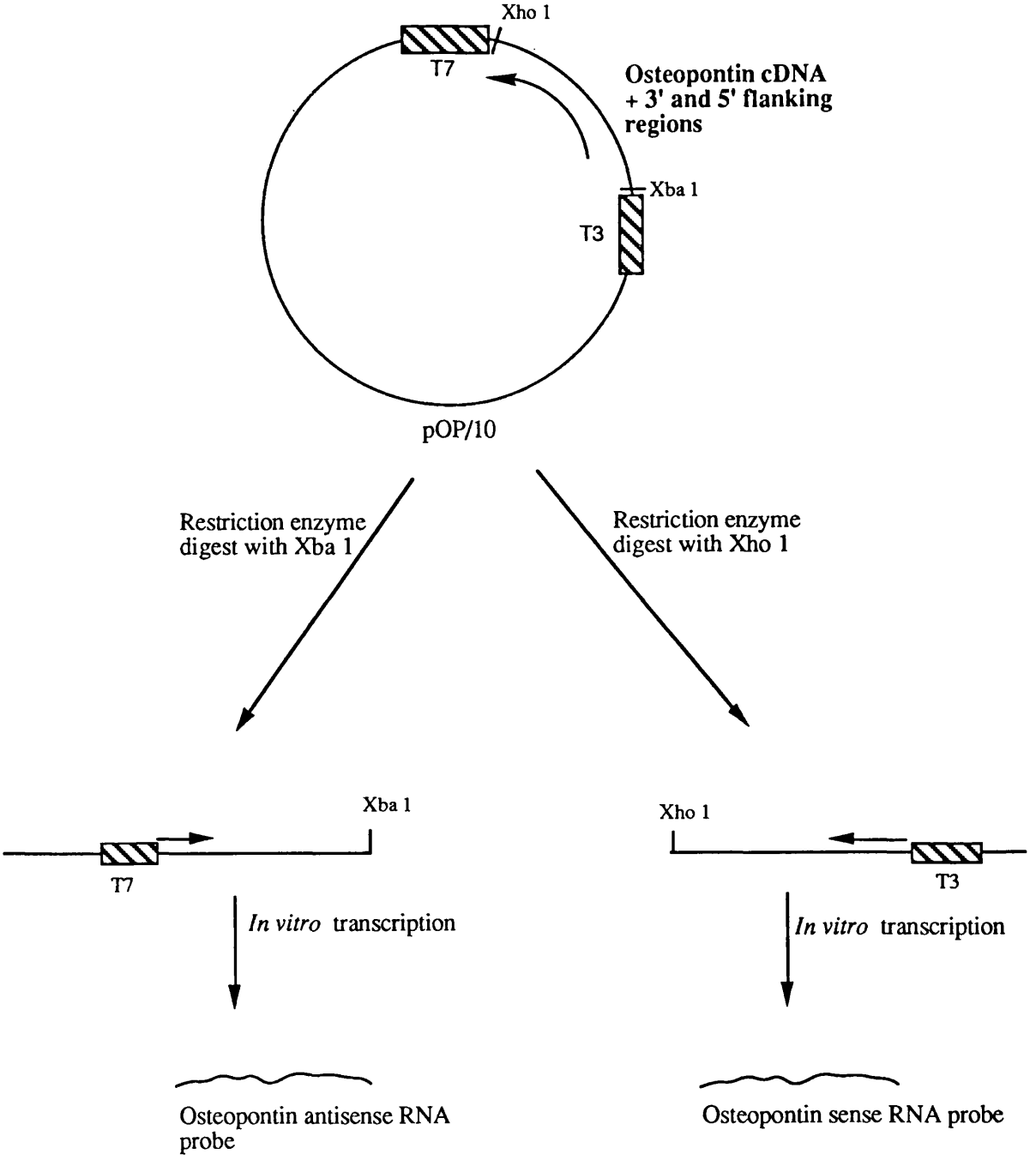
Sense and antisense RNA probes for osteopontin were prepared as follows. Preparations of the pOp/10 plasmid were linearized with the restriction enzymes Xba1 or Xho 1, to generate two linear templates each containing a T7 or T3 RNA polymerase site as shown in fig VII.1. Antisense RNA transcripts were generated with T7 RNA polymerase from the first template using the Manheim Boeringer *in vitro* transcription

kit. Similarly sense transcripts (to be used as a negative control) were generated from the other template using T3 RNA polymerase. After the synthesis of the RNA probe, the DNA template was removed using 2 units of DNAse I (RNAse free) at 37°C for 15 mins. This step was necessary, otherwise the DNA template could compete for RNA binding sites within the sections. The specific activity of both the antisense and sense RNA probes was assessed as described in chapter II, and both probes were analysed by vertical gel electrophoresis to confirm the majority of transcripts were of full length.

The *in situ* hybridization of the osteopontin RNA probes was undertaken as previously described in chapter II. Since the osteopontin cDNA template was approximately 1500 bp, the RNA probe gave rise to a greater degree of background binding than the smaller (300bp) probes for IL-1β and TGFβ. To reduce this non specific binding to an acceptable level, an additional high stringency wash of 1xSSC /50% formamide at 40°C was performed after the hybridization step.

Alkaline phosphatase and TRAP staining was undertaken as described in Chapter II.

Figure VII.1. Generation of osteopontin RNA probes.



VII.4 RESULTS

The three main stages of osteophyte development; cartilage formation and calcification, woven bone formation and remodelling, and lamellar bone formation are described in chapter VI (see figure VI.3). The profile of osteopontin expression by human bone cells is described in the following section at these stages of osteophyte development.

CARTILAGE FORMATION AND CALCIFICATION

The primary phase of osteophyte development involves the calcification and remodelling of cartilage. During these initial stages of cartilage calcification (fig. VII.2a), low osteopontin expression was detected in the newly recruited osteoblasts (fig VII.2b). Figure VII.3a shows a histological section of an area of remodelling cartilage; areas of proliferating, resting and hypertropic cartilage are present, together with a zone of calcified cartilage. Low levels of expression were detected in mature chondrocytes (fig VII.3b), but in areas of calcifying cartilage no expression was detected (fig VII.3b). In contrast, areas of differentiating fibrocartilage cells at the periphery of the osteophyte (fig VII.3a) expressed high levels of osteopontin (fig VII.3c). Fibrocartilage cells differentiating from the connective tissue (fig.VII.4a), also showed high osteopontin expression (fig. VII.4b).

In common with the expression profile of osteoblasts, low expression was detected in osteoclasts (fig. VII.5b), during the initial stages of cartilage resorption (fig VII.5c).

WOVEN BONE FORMATION AND REMODELLING

The residual calcified cartilage was remodelled into woven bone by osteoclastic resorption followed by bone formation. In contrast to the initial stage of cartilage calcification distinct clusters of osteoclasts were observed to express very high levels of osteopontin at sites of active woven bone remodelling (fig. VII.6a). The expression of osteopontin by osteoclasts was examined in several different environments. It was

demonstrated that osteopontin expression was not restricted to resorbing osteoclasts, as it was also detected in osteoclasts distant from the bone surface (fig. VII.7a and VII.7b).

Osteopontin expression was also investigated in the osteoclastoma tissue; figure VII.8a illustrates the histology of one of the osteoclastomas, and shows the osteoclasts surrounded by stromal and mononuclear cells. The entire population of osteoclasts in the osteoclastoma tissue (fig. VII.8b) were shown to express high levels of osteopontin mRNA, further confirming the observation that a bone environment was not necessary for expression.

In areas of newly forming intramembranous bone (fig. VII.9a) osteoblasts expressed high levels of osteopontin mRNA (fig VII.9b). High osteopontin expression was also particularly evident in osteoblasts that were differentiating from the surrounding connective tissue (fig. VII.10a) at the periphery of the forming osteophyte. Figure VII.10b shows the extremely high levels of osteopontin mRNA expressed in such an area.

The expression of osteopontin by the osteoblasts was shown to be dependent on their stage of differentiation. Osteoblasts that were plump and apposed to thick osteoid on the bone surface (fig. VII.11a) expressed high levels of osteopontin (fig. VII.11b). With the progression from cuboidal active osteoblasts (fig VII.12a) to flattened quiescent lining cells osteopontin expression decreased markedly (fig. VII.12b).

One very unusual finding was that populations of mononuclear cells within resorption sites (fig. VII.13a) expressed high levels of osteopontin mRNA (fig. VII.13b). This pattern of expression was seen in several resorption lacunae (another example is shown in figure VII.14 a,b and c). The identity of the mononuclear cells was not determined but they were unlikely to be osteoblasts as they were not co-localized with alkaline phosphatase activity.

LAMELLAR BONE FORMATION

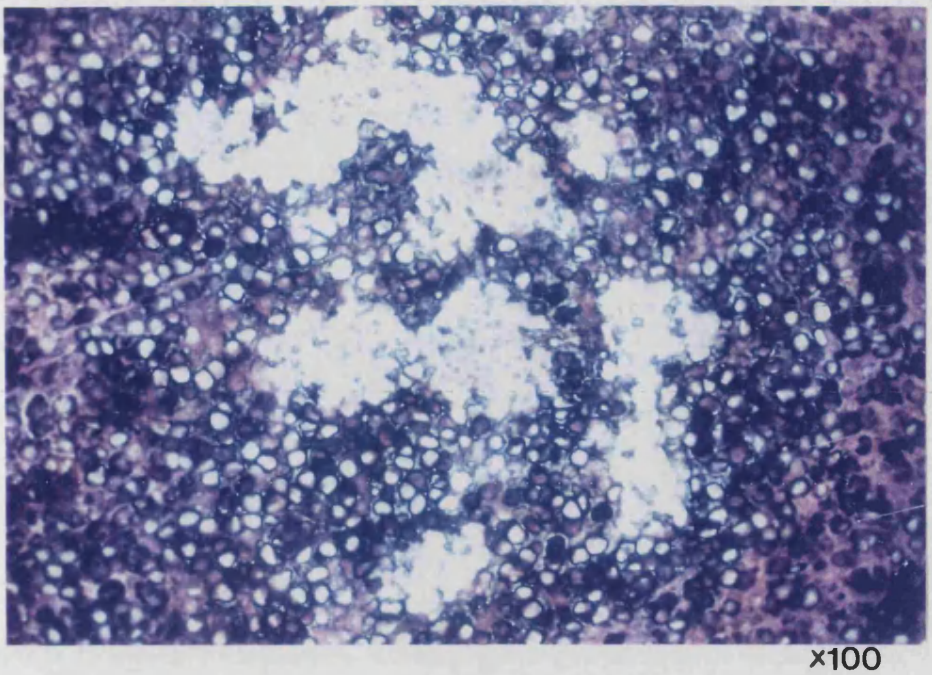
In areas of lamellar bone the expression of osteopontin seemed to be down-regulated. Osteopontin expression could only be detected in occasional osteoclasts as shown in fig. VII.14. Distinct populations of osteoblasts in lamellar bone expressed high levels of osteopontin (fig.VII.16), but the majority of cells showed very little osteopontin expression .

Therefore in summary the expression of osteopontin was analysed in twelve osteophytes in four separate experiments and four different osteoclastomas. Osteopontin expression was detected in chondrocytes, osteoblasts, and osteoclasts as shown in Table VII.1. The level of expression was dependent on several factors including; the environment of the bone cells , and the stage of differentiation of each cell type.

VII.5 FIGURES

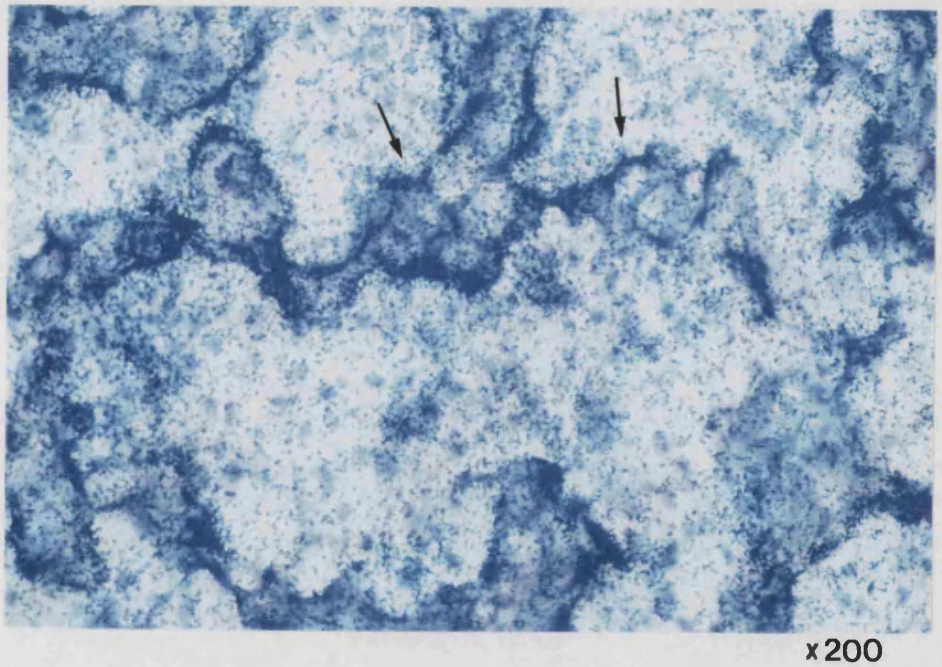
Figure VII.2a An area of calcifying cartilage.

Figure VII.2b. Very low levels of osteopontin expression detected in newly recruited osteoblasts (arrows).



×100

FIG 2a



x200

FIG 2b

Figure VII.3a Initial stages of cartilage calcification; including areas of proliferating and resting chondrocytes, hypertropic chondrocytes, together with a zone of calcified cartilage. The cartilage is apposed to an area of newly differentiated fibrocartilage cells (arrow).

Figure VII.3b Osteopontin expression is detected in immature chondrocytes, but very little expression was seen in the calcified cartilage zone. Note high expression in fibrocartilage cells.

Figure VII.3c Negative control showing lack of binding of the sense transcript.

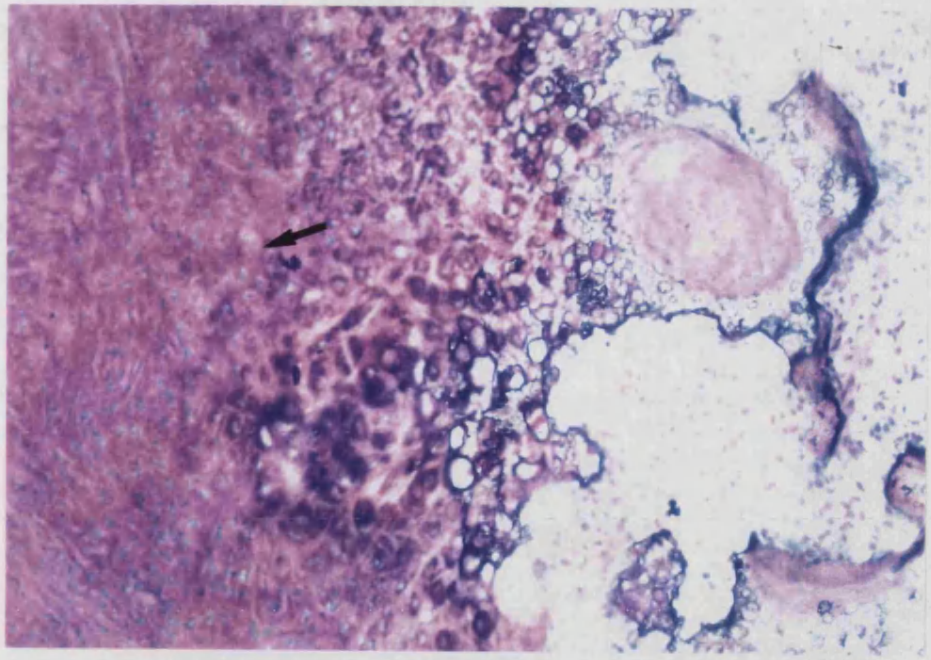


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FIG 3a

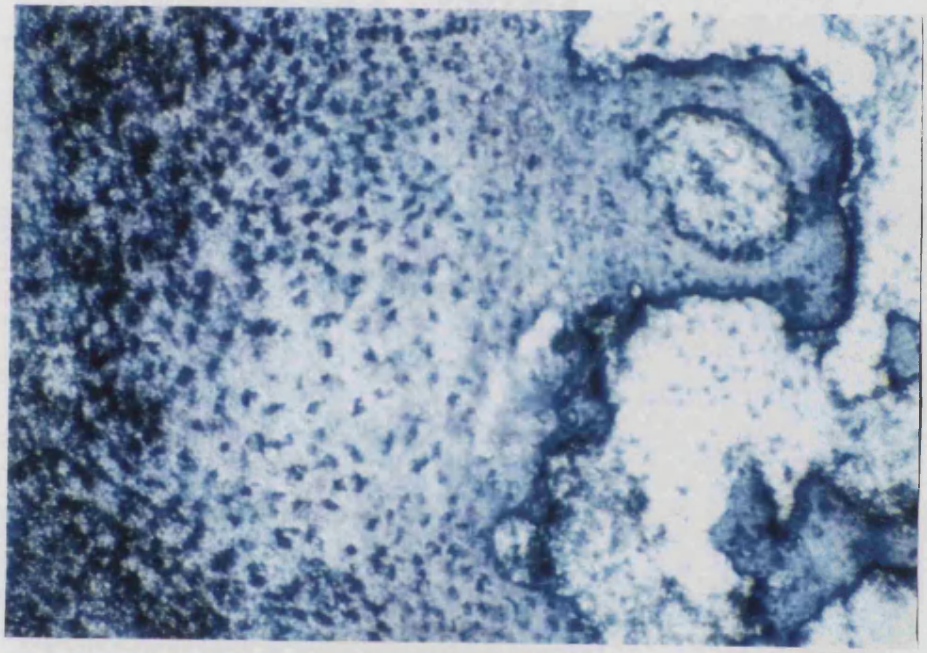

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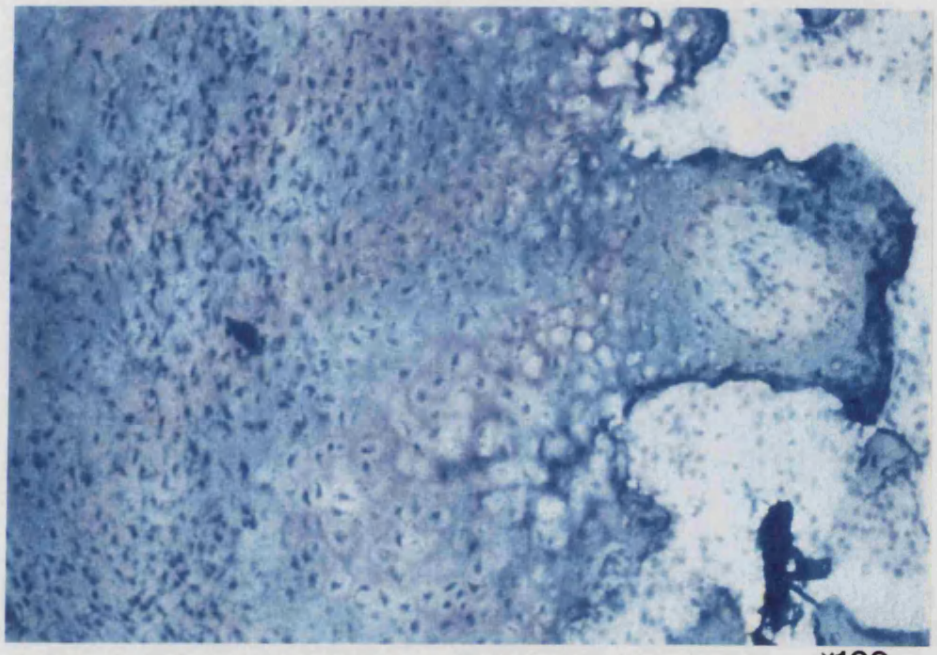
FIG 3b



x100

FIG 3c

Figure VII.4a Histology of an area of differentiating fibrocartilage cells (arrows).

Figure VII.4b High expression of osteopontin by fibrocartilage cells

Figure VII.4c Sense RNA probe negative control

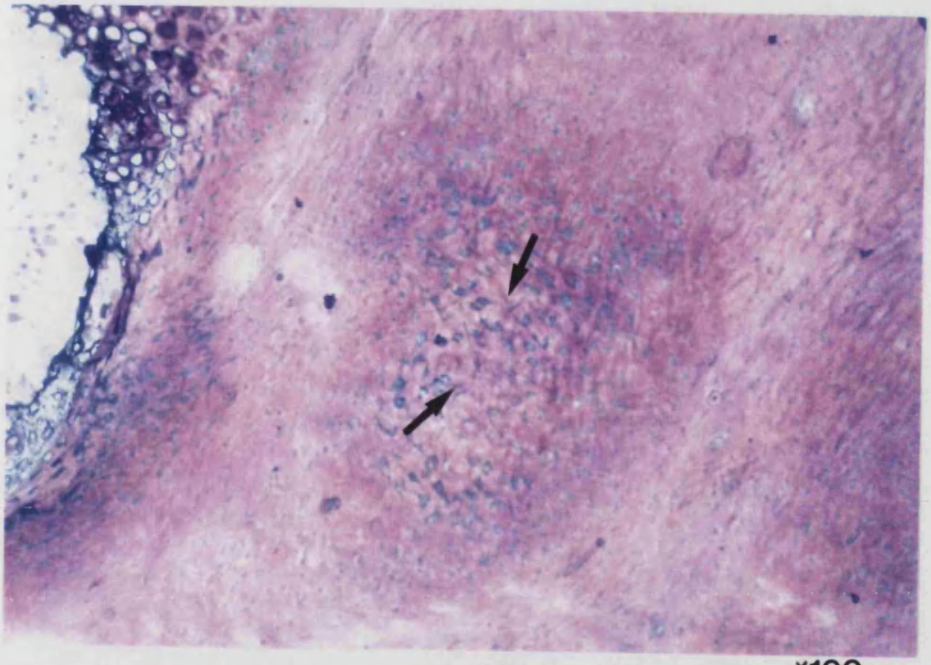


FIG4a

x100

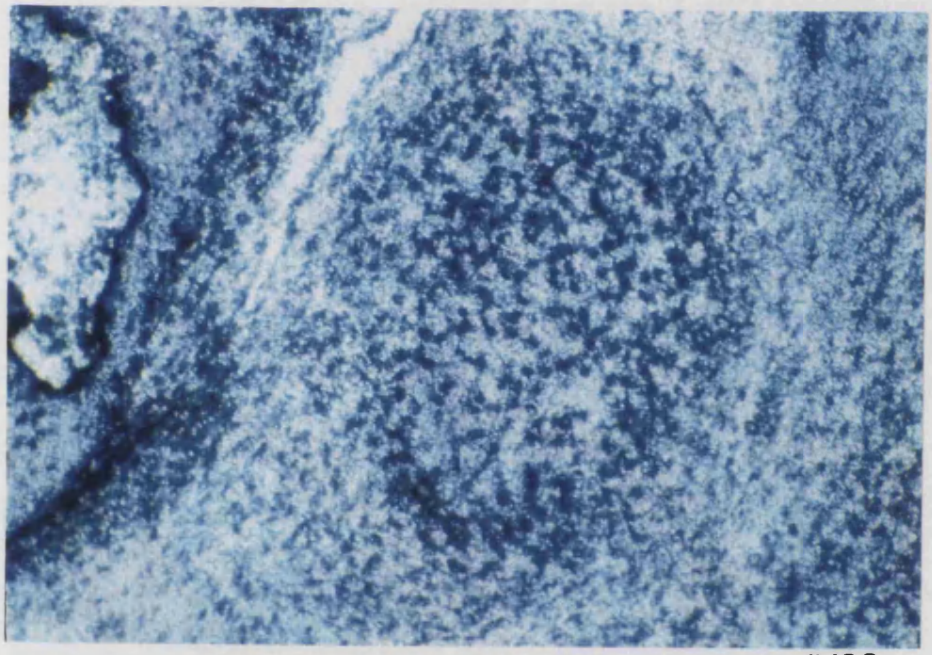


FIG4b

x100

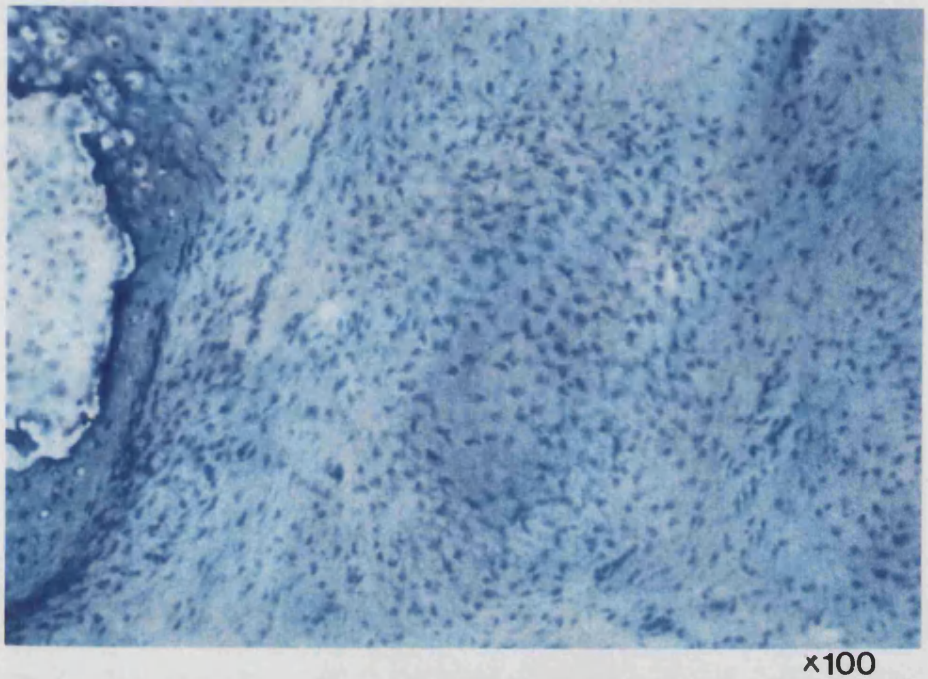


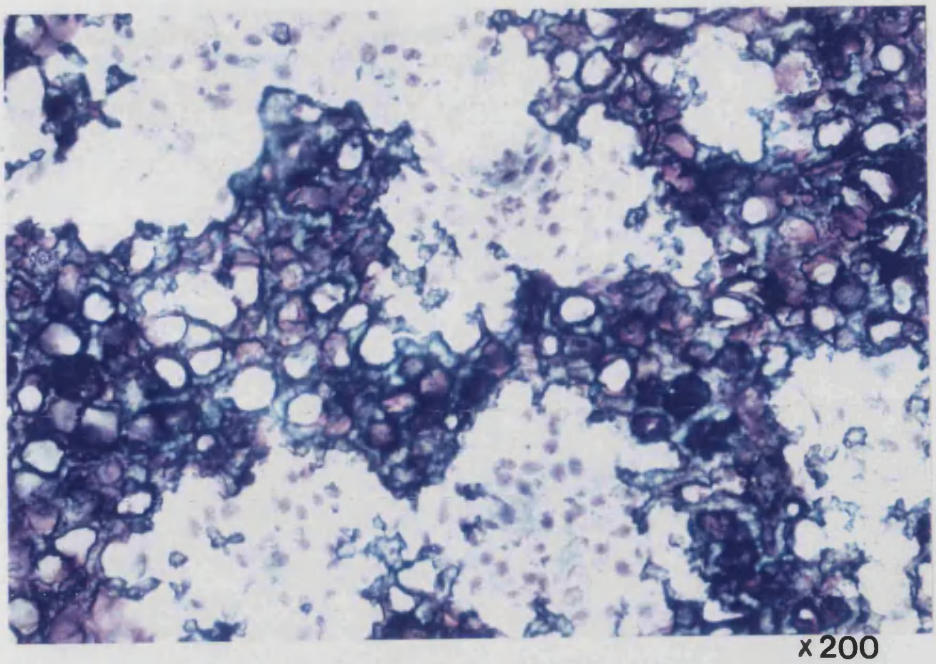
FIG4c

x100

Figure VII.5a An area of residual calcifying cartilage.

Figure VII.5b. Osteopontin expresion is undetectable in newly recruited osteoclasts (arrows).

Figure VII.5c Serial section reacted for TRAP activity to highlight osteoclasts (arrows).



x200

FIG5a

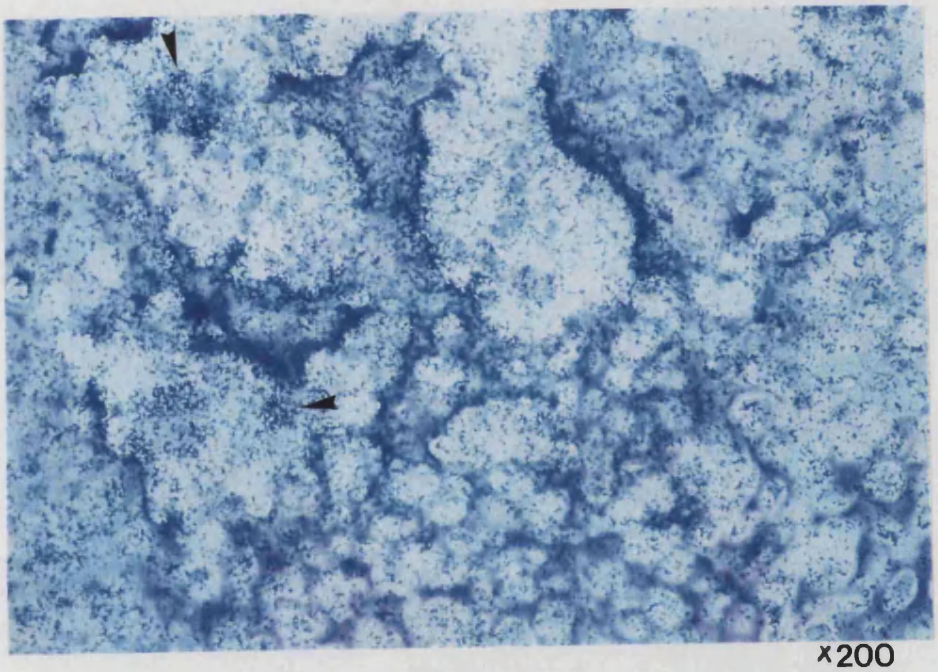


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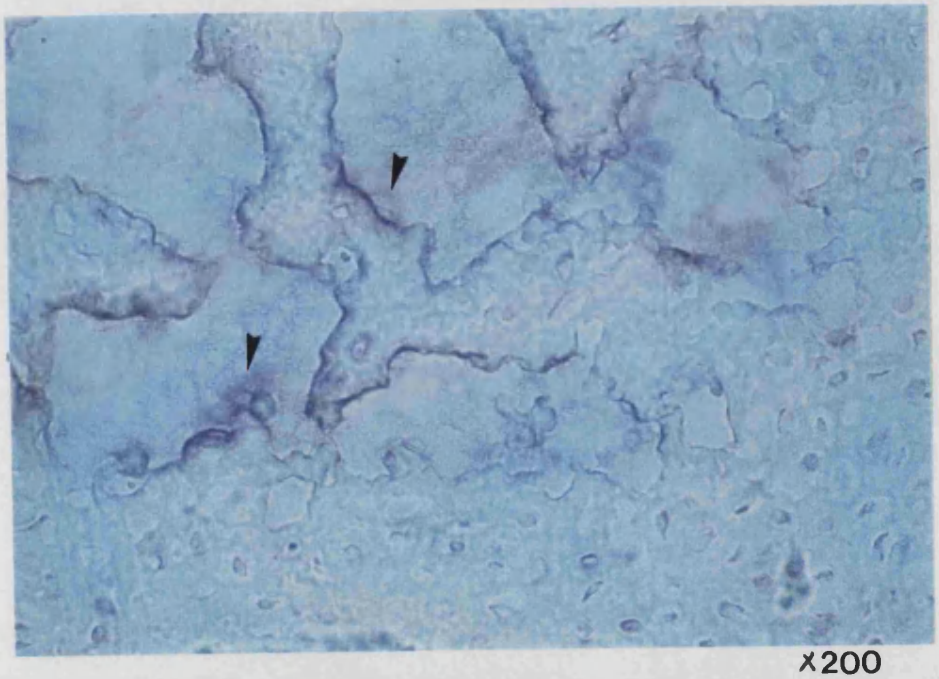
FIG5b

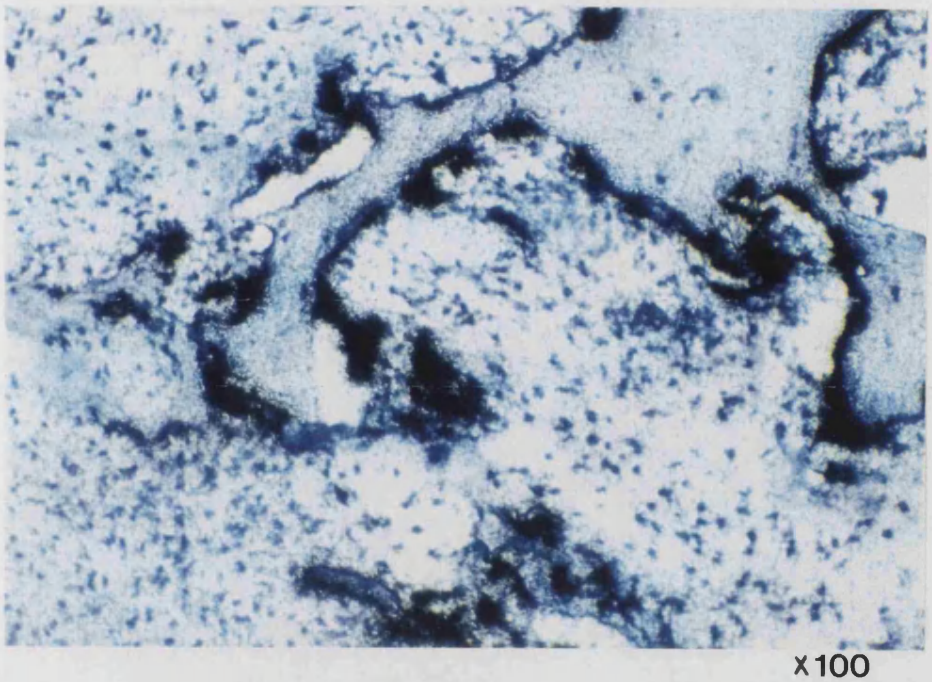


x200

FIG5c

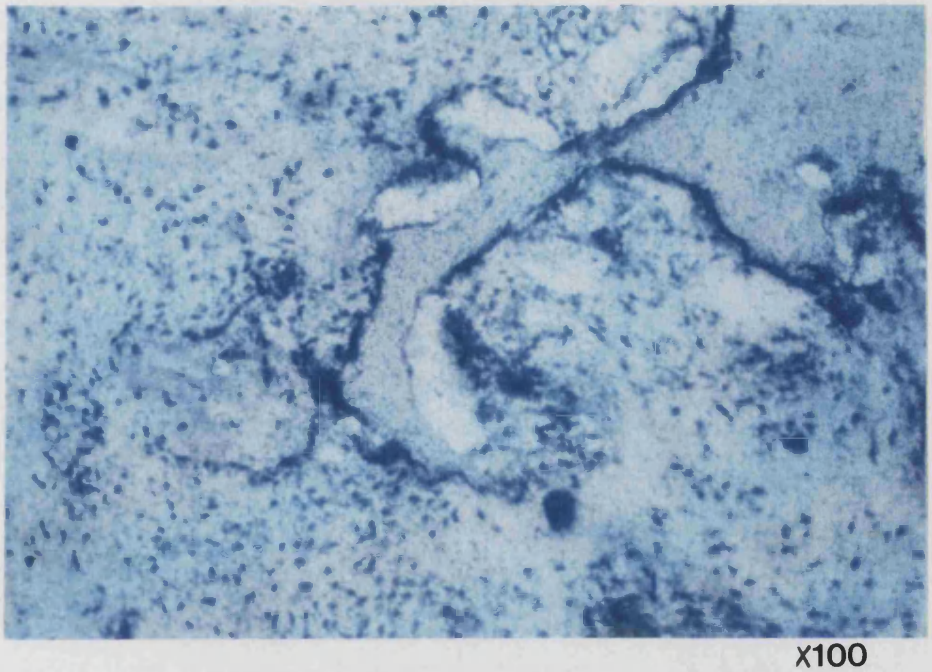
Figure VII.6a At sites of woven bone remodelling, clusters of osteoclasts expressed high levels of osteopontin.

Figure VII.6b Negative control showing low level of background hybridization.



x100

FIG6a



x100

FIG6b

Figure VII.7a An osteoclast away from the bone surface.

Figure VII.7b Osteoclast distant from the bone surface expressing high levels of osteopontin mRNA.

Figure VII.7c Sense RNA probe negative control.

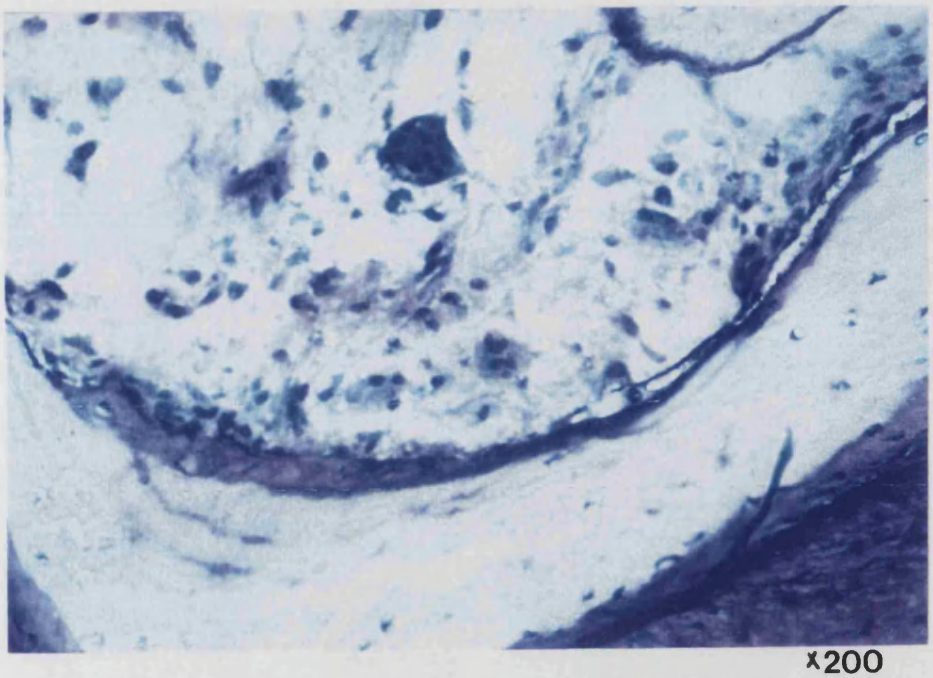


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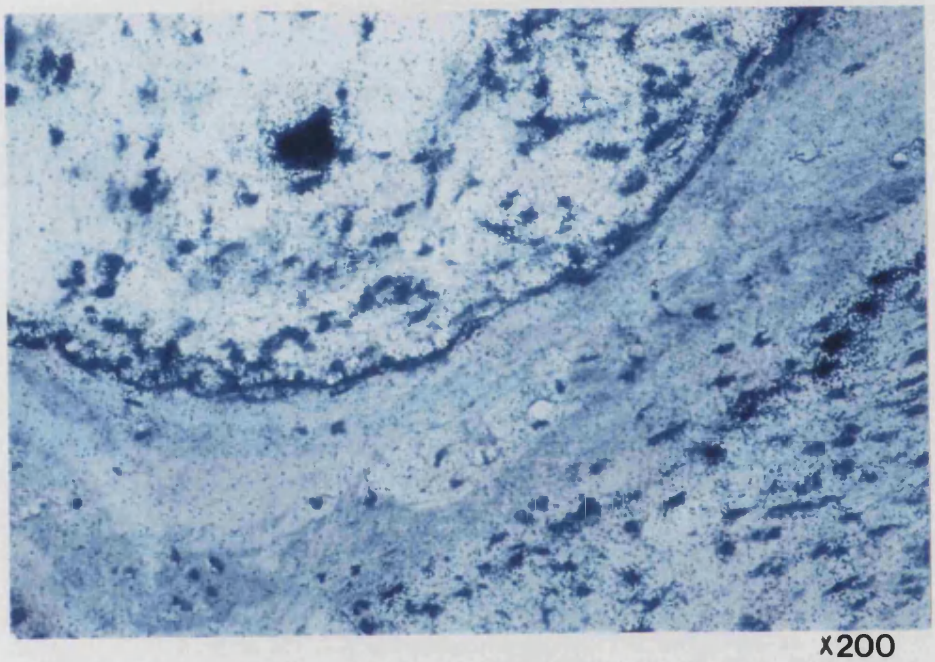
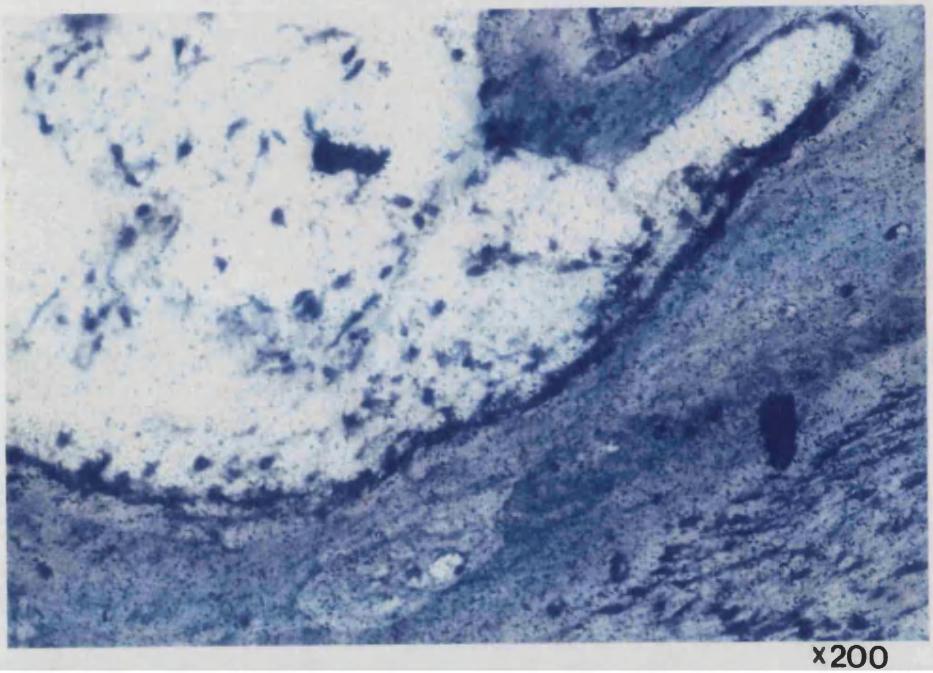
FIG7a



x200

FIG7b



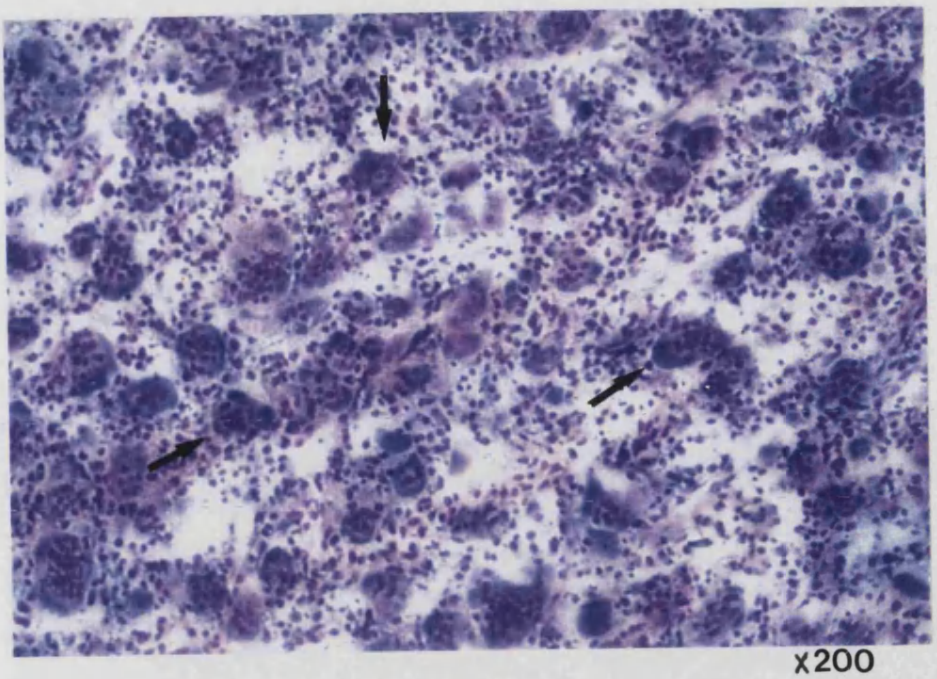
x200

FIG7c

Figure VII.8a Histology of a section of osteoclastoma showing osteoclasts (arrows) and stromal cells.

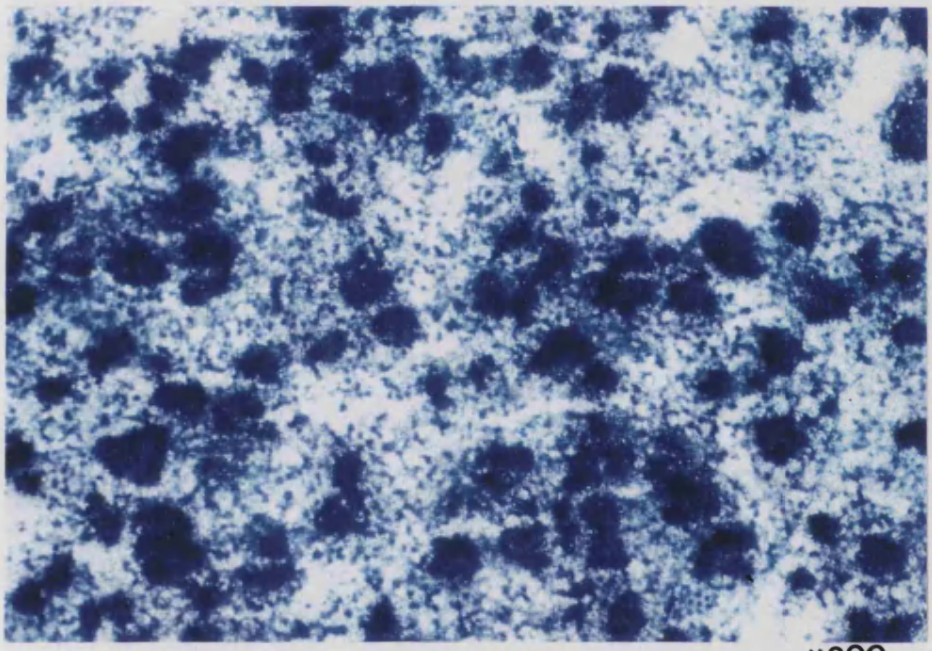
Figure VII.8b High osteopontin expression in osteoclasts, note low expression in occasional mononuclear cells.

Figure VII.8c Sense RNA probe negative control.



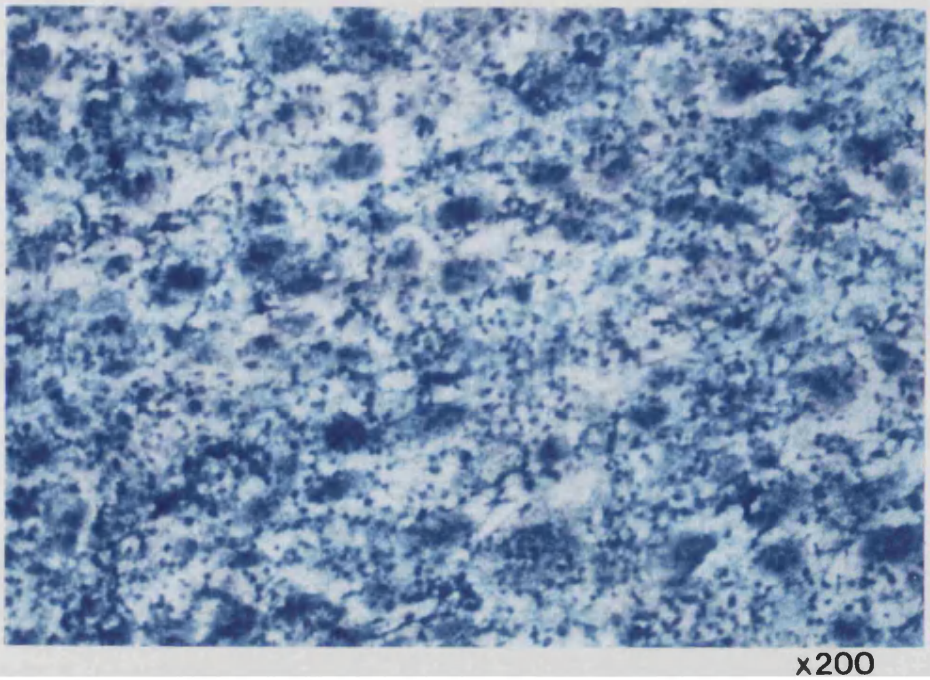
x200

FIG8a



x200

FIG8b

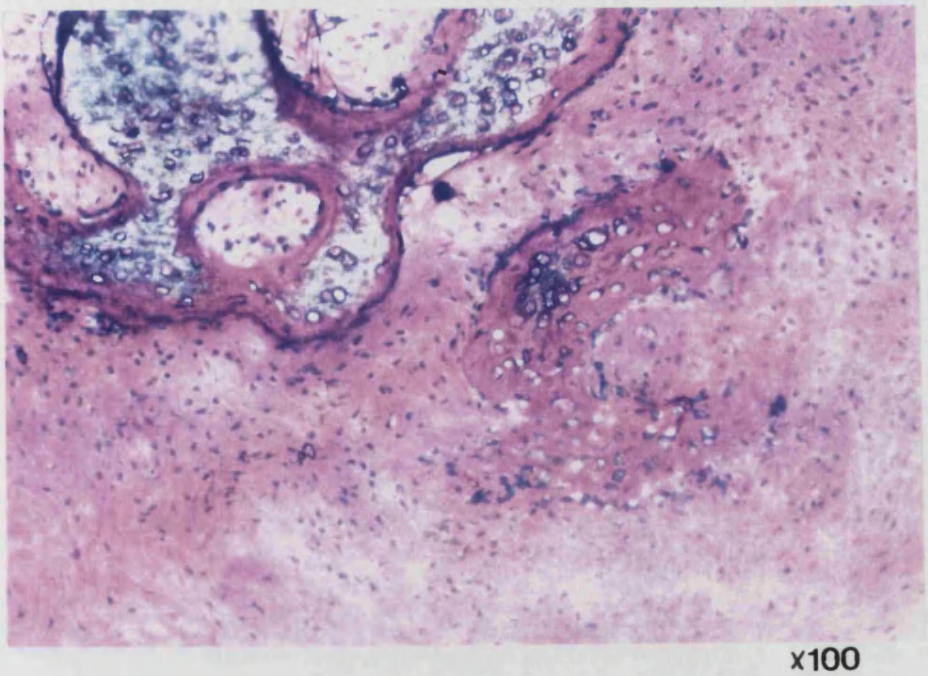


x200

FIG8c

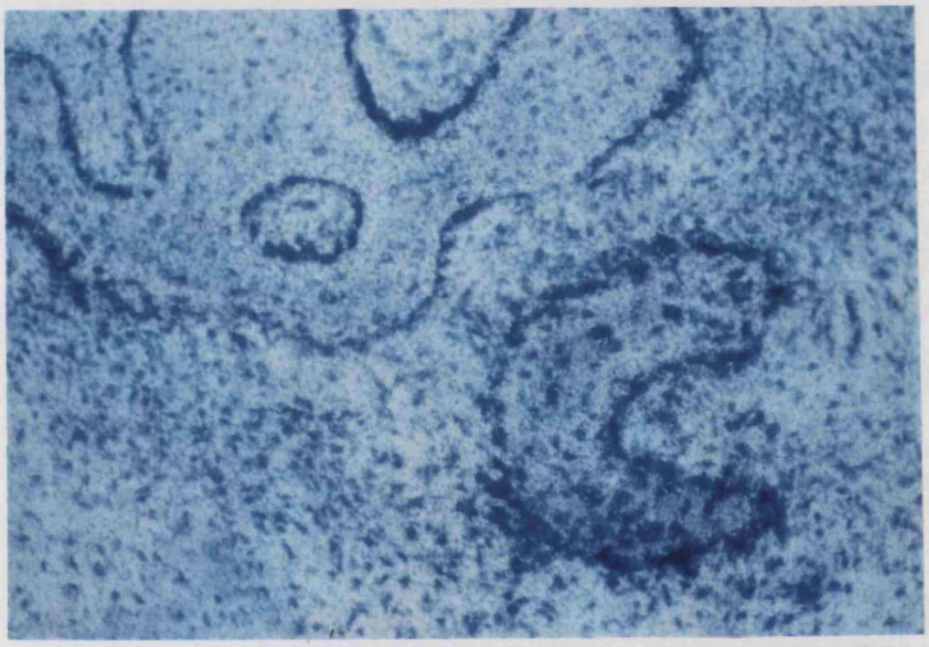
Figure VII.9a. An area of intramembranous bone formation where osteoblasts have differentiated from the mesenchymal tissue, and deposited bone matrix.

Figure VII.9b High expression of osteopontin by osteoblasts in an area of intramembranous bone formation.



x100

FIG9a



x100

FIG9b

Figure VII.10a At the tip of the developing osteophyte, sites of diminished endochondral bone formation occur. Osteoblasts differentiate from the surrounding connective tissue and lay down bone directly onto the cartilage.

Figure VII.10b High osteopontin expression in osteoblasts in this area.

Figure VII.10c Negative control

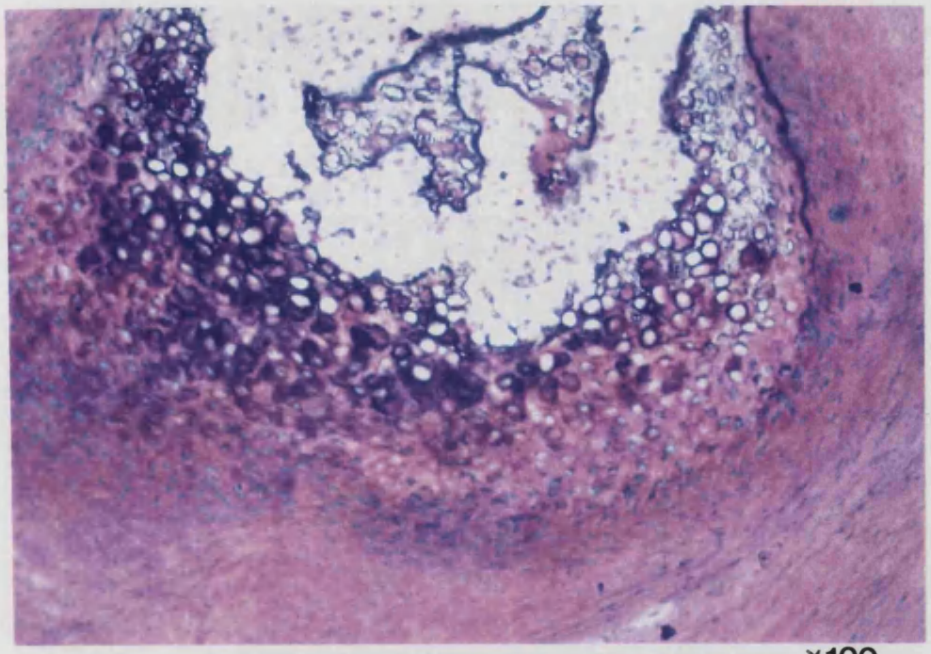


FIG10a

×100

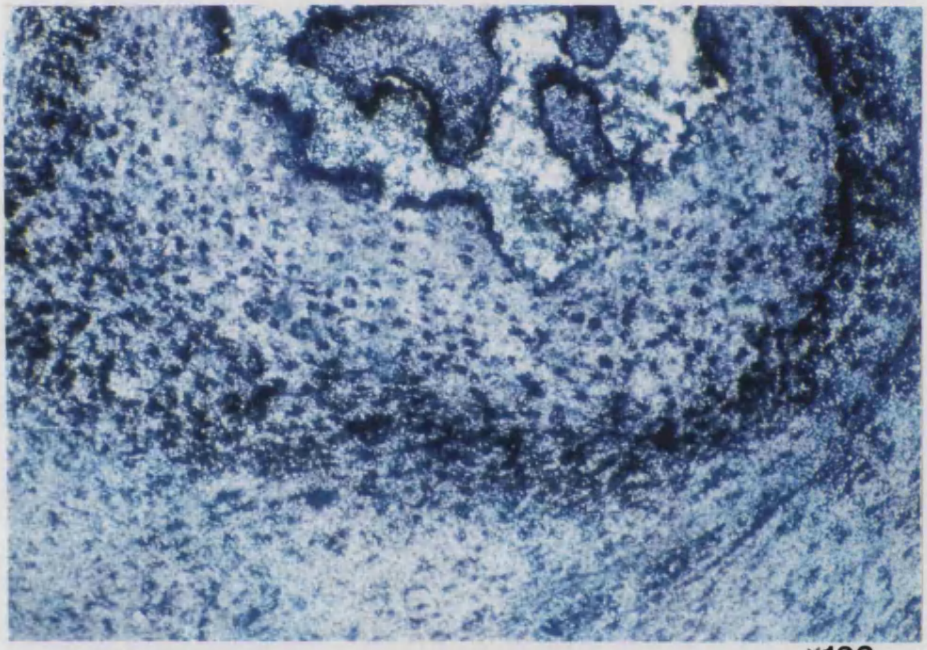


FIG10b

×100

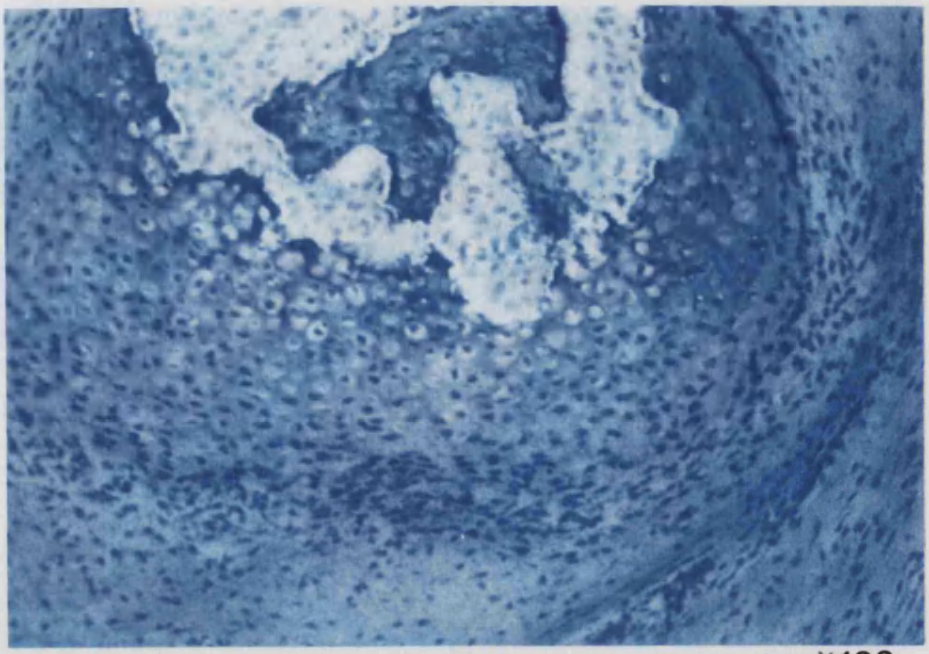
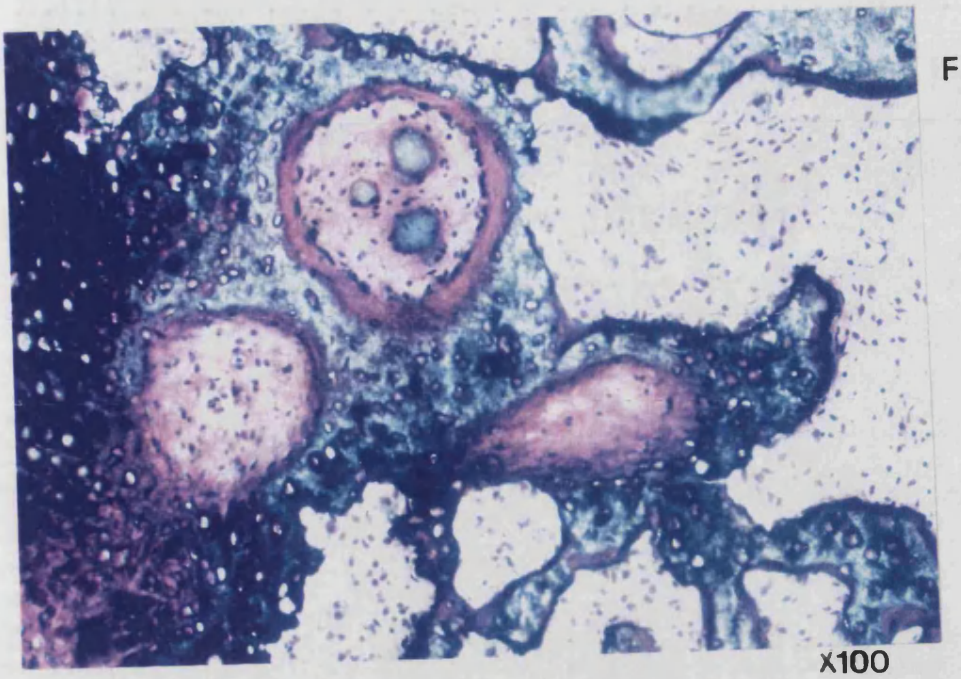


FIG10c

×100

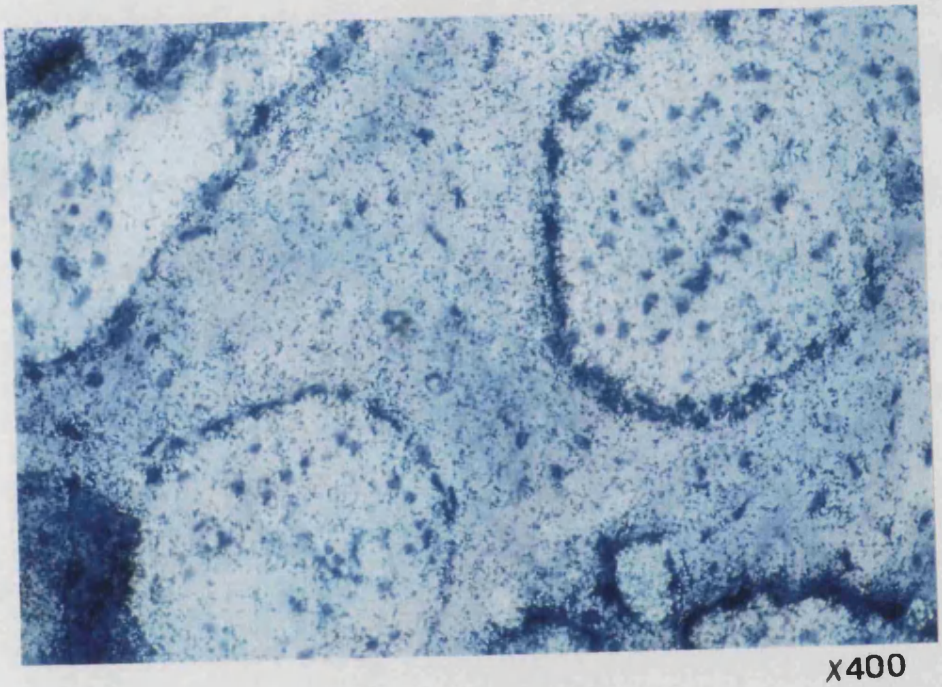
Figure VII.11a Osteoblasts actively laying down osteoid at sites of bone formation.

Figure VII.11b Osteoblasts express high levels of osteopontin. Note the upper bone forming surface with thicker osteoid shows higher expression than the lower area.



x100

FIG11a



x400

FIG11b

Figure VII.12a A phase of bone formation by osteoblasts. The histological changes from left to right are as follows: Plump active osteoblasts (arrow) to flatter quiescent osteoblasts on mineralised osteoid.

Figure VII.12b Osteopontin mRNA expression by osteoblasts, note plump active osteoblasts express higher levels of osteopontin than flatter more quiescent osteoblasts.

Figure VII.12c Sense RNA probe negative control

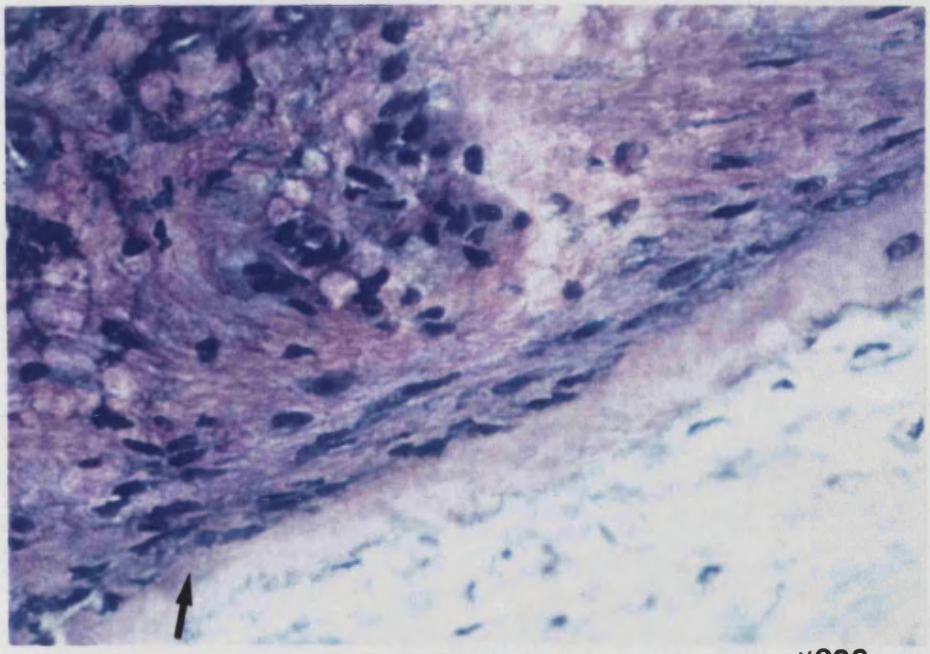


FIG12a

×200

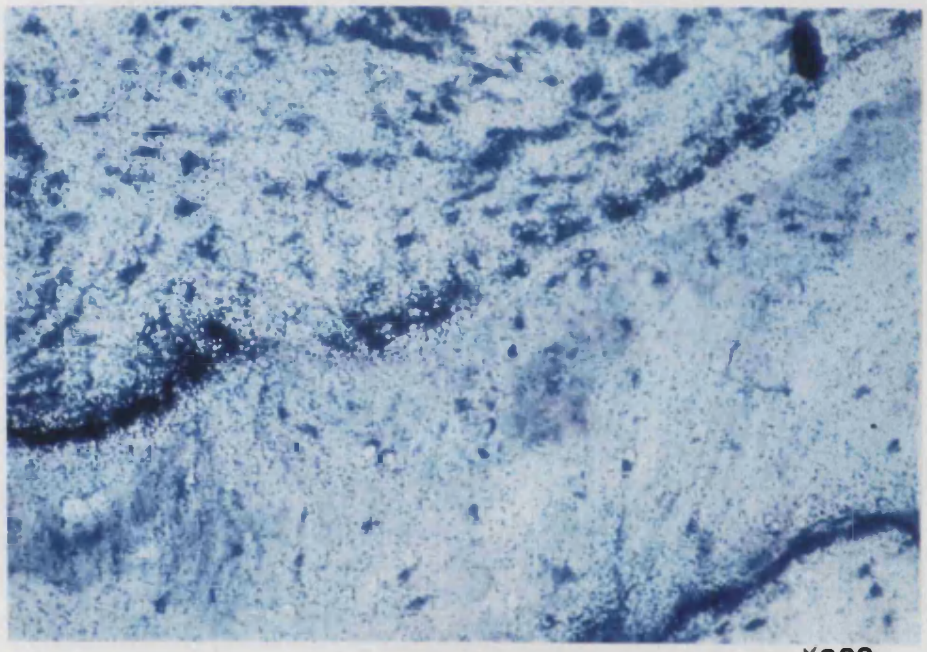


FIG12b

×200

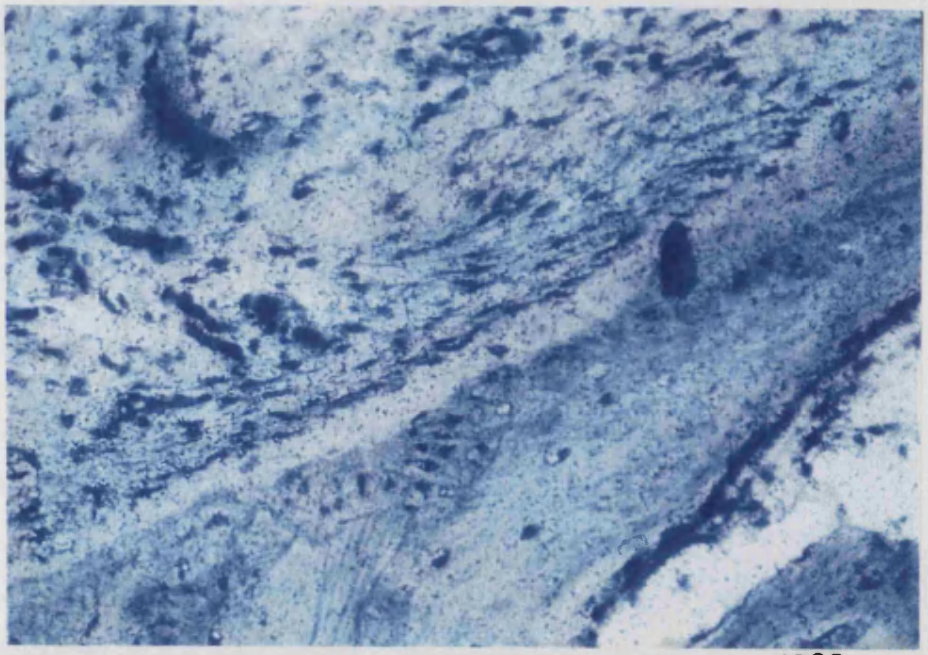


FIG12c

×200

Figure VII.13a Low magnification of mononuclear cells in a resorption site.

Figure VII.13b High osteopontin expression in mononuclear cells in resorption lacunae.

Figure VII.13c Sense RNA probe negative control

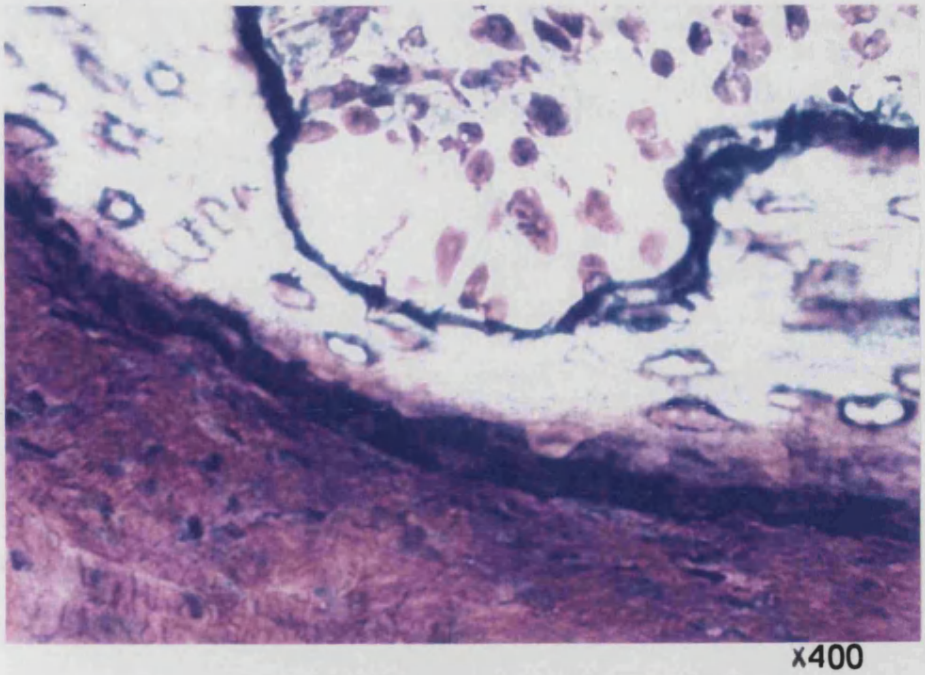


FIG13a

x400

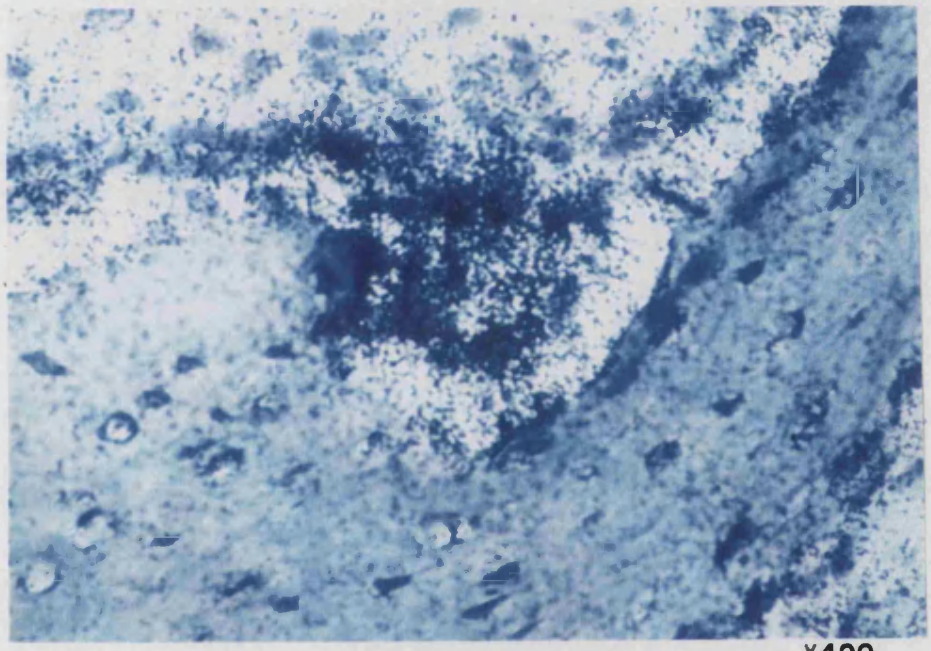


FIG13b

x400

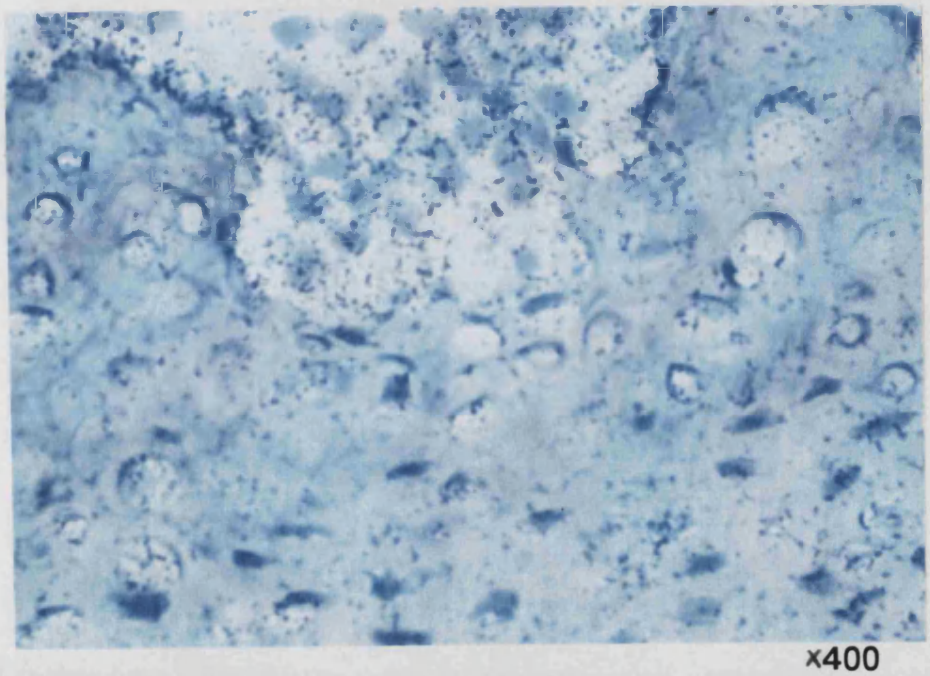


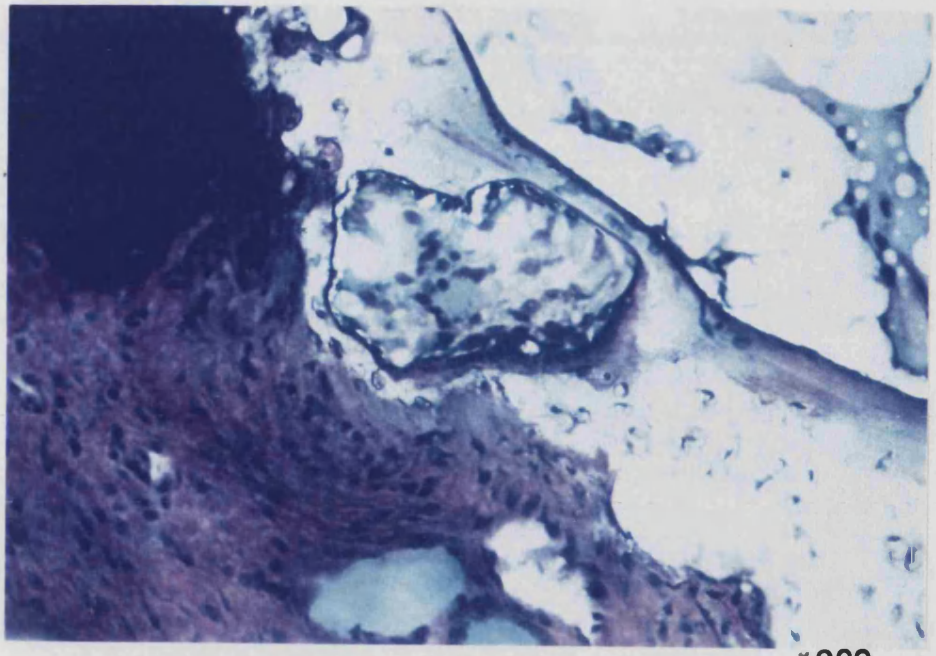
FIG13c

x400

Figure VII.14a High magnification of another resorption site.

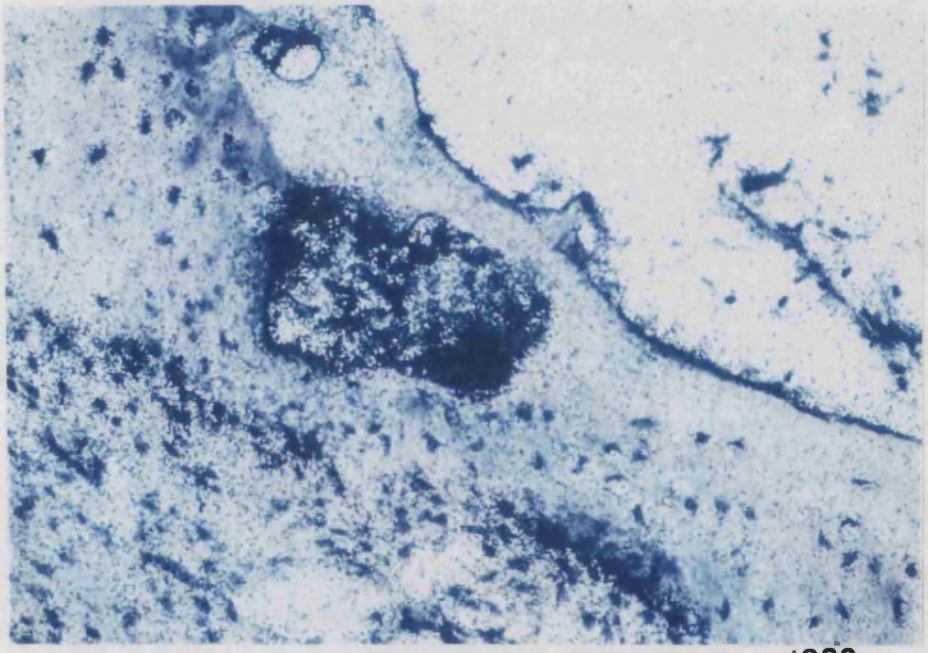
Figure VII.14b High expression in mononuclear cells in resorption lacunae.

Figure VII.14c Sense RNA probe negative control.



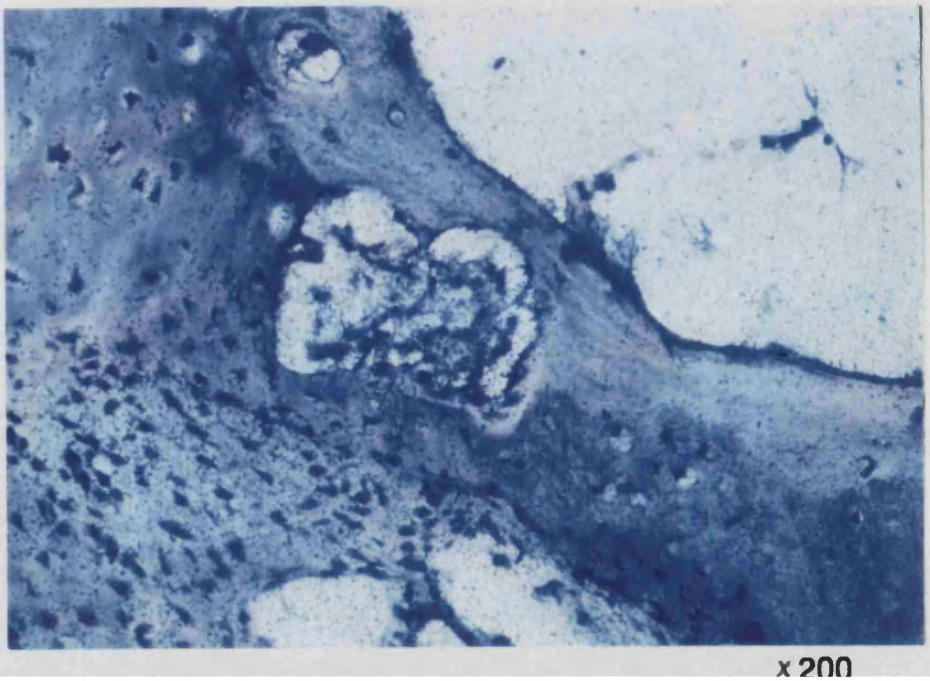
x200

FIG14a



x200

FIG14b



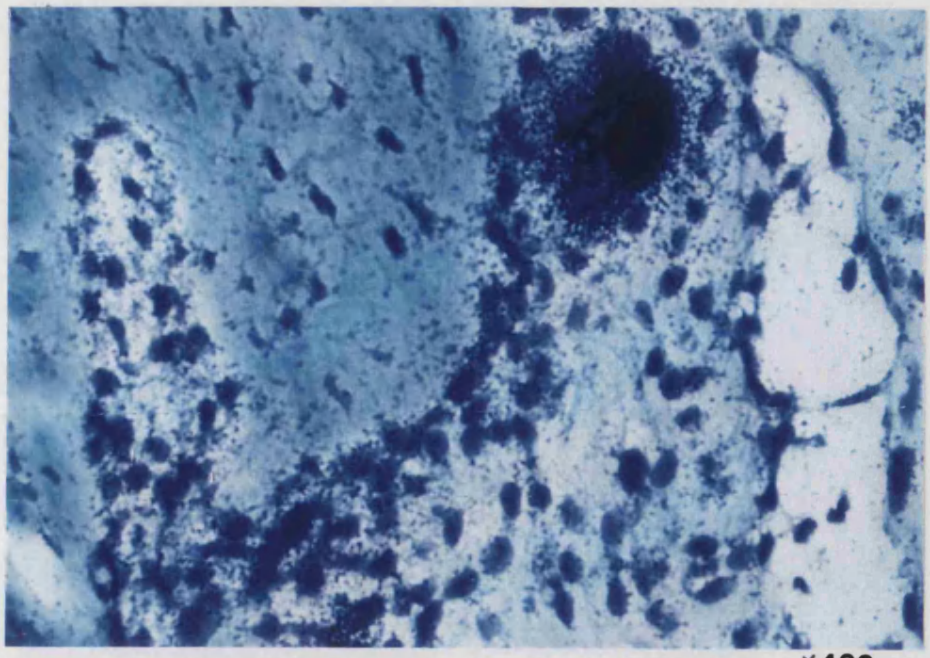
x200

FIG14c

Figure VII.15a Occasional osteoclasts expressing osteopontin on surfaces of lamellar bone.

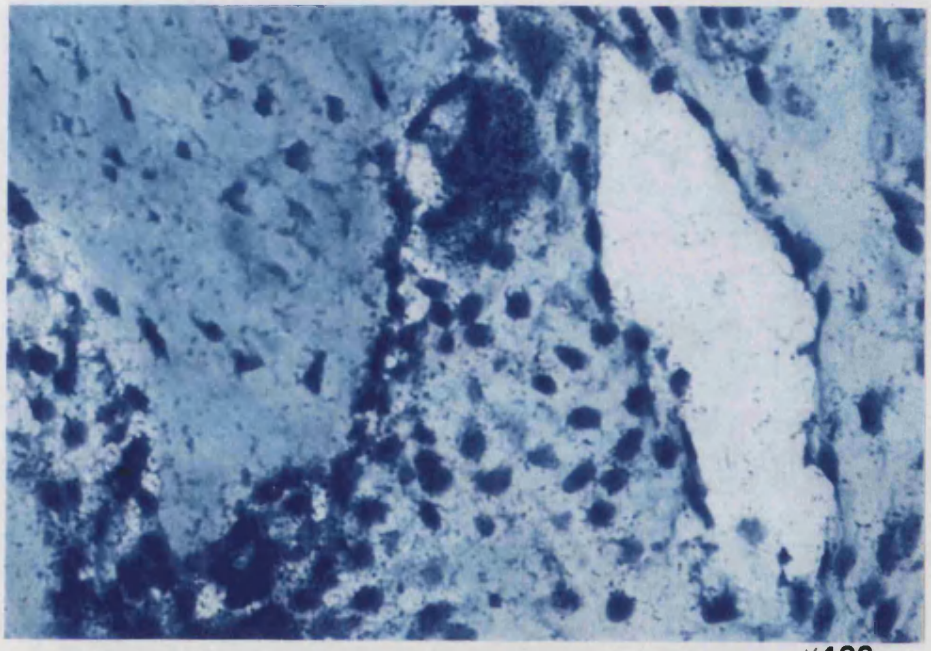
Figure VII.15b Sense RNA probe negative control.

Figure VII.16a Distinct population of osteoblasts expressing osteopontin in lamellar bone,
the majority of cells show very little osteopontin expression. Note also weak expression in some osteocytes (arrows).



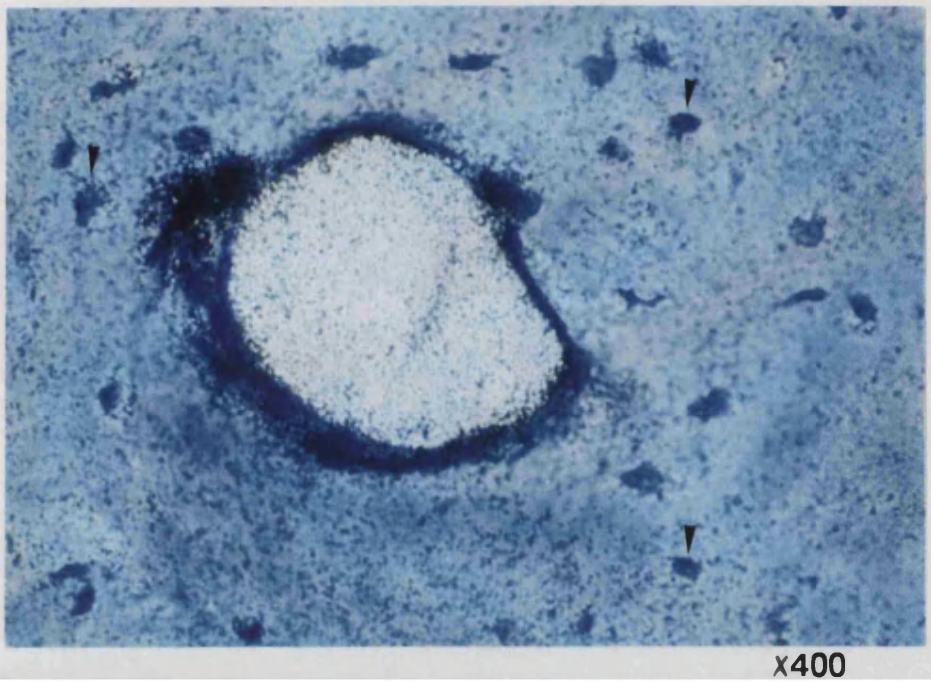
x400

FIG15a



x400

FIG15b



x400

FIG16a

VII.6 DISCUSSION

The expression of osteopontin mRNA has been demonstrated in a variety of different cell types in human bone as summarized in Table VII.1. This study has confirmed previous data from cell culture experiments, which suggested that osteoblasts were the main osteopontin producing cell type. The expression of osteopontin by osteoblasts was dependent on the maturation stage of the cell. Differentiating osteoblasts expressed high levels of osteopontin, in contrast to quiescent lining cells where very little expression was detected. These observations suggest that osteopontin is synthesized at quite an early stage in bone matrix formation, possibly before the onset of mineralisation. These observations are supported by Mark et al.(1987) who detected osteopontin expression in a preosteblast population in rat bone. This expression profile is similar to many matrix components including osteonectin (Bianco et al.1988).

High levels of osteopontin expression were detected in osteoblasts in areas of intramembranous bone formation. This expression probably reflects the fact that during intramembranous bone formation there is a large "burst" of matrix protein expression to form islands of osteoid. It would be interesting to monitor the expression of other bone matrix proteins, particularly osteocalcin, during intramembranous bone formation.

It is interesting to note that certain populations of chondrocytes also expressed osteopontin. The expression decreased with maturation of the cartilage: differentiating fibrocartilage cells expressed high levels of osteopontin mRNA whilst chondrocytes in calcifying cartilage expressed no osteopontin mRNA. This expression profile was very similar to that observed for osteoblasts. Since chondrocytes and osteoblasts are thought to arise from a common precursor they may share the expression of some matrix proteins.

Presumably there must be a different regulatory mechanism for the expression of osteopontin by osteoblasts and other connective tissue cells, since during the initial

stage of cartilage formation and calcification, high expression was detected in fibrocartilage cells but not in newly recruited osteoblasts. It is possible that some of the non collagenous proteins of bone perform some of their functions within the cells, or in non mineralised matrix long before calcification. Alternatively osteopontin may facilitate cell : cell interactions by binding to the integrins on the cell surface. This idea is supported by the finding that many transformed tumourogenic cell lines express aberrantly high levels of 2ar, the mouse equivalent of osteopontin (Craig et al.1988).

The most surprising feature of osteopontin expression was the large amount of mRNA detected in certain populations of osteoclasts; as it has been previously assumed that osteoblasts were the major synthetic cell for osteopontin. Osteopontin has been postulated to be a ligand for the vitronectin receptor ($\alpha_v\beta_3$) (Reinholt et al.1990) which is expressed on the cell membrane of osteoclasts from many species (Davies et al.1989). Zambonin-Zallone et al. (1988) demonstrated that the vitronectin receptor was particularly enriched in dot-like adhesion structures called podosomes. These structures are thought to facilitate the adhesion to bone via a specific matrix recognition event. Furthermore osteopontin has been shown to be highly enriched at regions of the bone surface where osteoclasts were anchored. Therefore it is likely that osteoclastic attachment to the bone surface is via osteopontin-vitronectin receptor interactions. Studies with monoclonal antibodies to the vitronectin receptor have shown that the perturbation of vitronectin receptor binding blocks osteoclastic resorption (Chambers et al.1986).

This study has shown that osteoclasts express osteopontin mRNA, suggesting that osteoclasts may facilitate their own attachment to bone, and are therefore not restricted to sites of osteoblastic osteopontin synthesis for attachment. The idea that osteoclast derived osteopontin is involved in attachment is further supported by the observation that osteoclasts only expressed osteopontin mRNA during the active woven bone resorption stage and lamellar remodelling, and not during the initial cartilage calcification resorption stage. Presumably this means that osteopontin synthesis was only required during the resorption of bone. Osteopontin expression was not

dependent on attachment to bone since high levels of osteopontin mRNA were detected in many osteoclasts distant from the bone surface. Furthermore, the entire population of osteoclasts in the osteoclastoma tissue expressed osteopontin mRNA. It is possible that the osteoclasts in the osteoclastoma tissue might not express osteopontin mRNA with the same expression profile as osteoclasts in bone, due to their aberrant environment. It must also be noted that *in situ* studies only highlight expression at a particular time point, therefore it is possible that osteoclasts distant form the bone surface which are expressing osteopontin may be preparing for resorption, or are in a post-resorptive phase.

Recently Bianco et al. (1991) demonstrated that osteoclasts in human fetal bone expressed both BSP mRNA and protein. BSP is thought to be a cell attachment factor and it is possible that osteoclasts express these two sialoproteins to facilitate attachment to the bone surface. The results of these studies have far reaching implications, as it has been previously presumed that sialoproteins located in osteoclasts were present as a result of phagocytic engulfment from the bone matrix.

The expression of osteopontin in certain mononuclear cells in resorption lacunae was unexpected, and it is tempting to speculate on the origin and function of these cells. It is possible for instance that their expression of osteopontin is a "post-resorptive" signal to recruit osteoblasts to initiate bone formation in the resorption lacunae. It has been reported that certain C terminal fragments of another bone matrix protein, osteocalcin, are chemotactic for osteoblasts (Lucas et al.1988). Perhaps osteopontin peptides share this property and encourage osteoblast recruitment to resorption lacunae. Alternatively these cells might be fission products of mature osteoclasts as recent data suggests that osteoclasts are " broken down " by phagocytic mononuclear cells after resorption has taken place (Video demonstration ICCRH meeting Copenhagen 1991). A panel of monoclonal antibodies specific for a range of cell types reacted to serial sections may identify these cells in the future.

In summary this study confirms that human osteoblasts are capable of expressing osteopontin mRNA in bone. Whilst osteoblasts may be the primary osteopontin

synthesizing bone cell, the data highlights the fact that other cell types in the bone microenvironment are capable of expressing osteopontin, including osteoclasts and "post-resorptive" mononuclear cells.

Table VII.1: Osteopontin mRNA expression in human bone and osteoclastoma tissue.

Tissue	Osteopontin expression
Cartilage	
Differentiating fibrocartilage	++
Fibrocartilage	+
Chondrocyte clusters	+
Mature chondrocytes	±
Hypertrophic chondrocytes	-
Calcified cartilage	-
Bone	
Differentiating osteoblasts	++
Osteoblasts forming intramembranous bone	++
Quiescent osteoblasts	-
Osteoblasts apposed to trabeculae	+
Osteoclasts resorbing cartilage	±
Osteoclasts remodelling bone	++++
Mononuclear cells in lacunae	+++
Osteoclastoma	
Osteoclasts	++++
Mononuclear cells	++

Chapter VIII: General discussion

The aim of this project was to characterise the expression of the cytokines TGFβ and IL-1β and the bone matrix protein osteopontin by human bone cells. This aim was largely achieved, and this study represents the first report of cytokine expression *in situ* in human bone. The following sections describe how the results obtained from this study compare with those from research groups in the same field. The pitfalls and limitations of the *in vitro* and *in situ* techniques will also be discussed. The role of the osteoclast in bone remodelling will be evaluated in the light of the novel data that was obtained in this study.

In vitro studies

The tissue culture experiments provided a simple system where the effect of exogenously added agents on cytokine expression could be studied. There are several advantages of such a simple system, the primary advantage being that the experimental design is very flexible and the effect of many different time points and concentrations of agent on cytokine expression can be evaluated. In addition, the experiments are relatively easy to perform and the model is a "clean" system where no unknown factors are present. One major disadvantage of this type of experiment is a certain variability between cells derived from different bone donors. The donors varied in age and sex, and a large number suffered from bone diseases such as osteoporosis and osteoarthritis. Whilst the same trends in cytokine expression were seen for all the bone cell populations studied, often the magnitude and time course of the responses differed. The results of the *in vitro* studies which investigated the modulation of TGFβ and IL-1β expression have been confirmed by other workers. Keeting et al. (1991) verified that human osteoblast-like cells express IL-1β mRNA, and that the transcription of this cytokine is induced by IL-1, TNFα and LPS treatment. Studies from the same group also confirmed that the expression of TGFβ mRNA by human osteoblast-like cells was

upregulated by systemic hormones, including PTH and 17-β oestradiol (Oursler et al. 1991).

It is important not to underestimate the limitations of *in vitro* studies. In a recent review, Nathan and Sporn (1991) proposed that the expression of cytokines by a variety of different cell types is highly influenced by the microenvironment of the cell. They coined the phrase "cytokines in context" to describe how the response of cells to cytokines could be markedly affected by the extracellular matrix in which most cells are embedded. It is reasonable to expect that the phenotype of human osteoblast-like cells may be altered during culture on a plastic surface. This change in environment could alter the whole cytokine expression profile of the cells.

A study has been undertaken to evaluate the effect on phenotype of culturing human osteoblast-like cells on different matrices. The expression of alkaline phosphatase, an important osteoblast secretory product, has been shown to be reduced by culturing these cells in collagen gels. Additionally, culture on a laminin substrate gave rise to a reduction of both alkaline phosphatase and osteocalcin production by human osteoblast-like cells (J. Clover , personal communication).

Human osteoblast-like cells in culture have been shown to express α1, α2, α3 and β1 integrin subunits (J. Clover, personal communication). These subunits are thought to facilitate attachment to a variety of different substrata including collagen, laminin and fibronectin. There is evidence that adherence to matrix may induce cells to produce cytokines in certain environments. Examples include the observation that GM-CSF transcription is induced in macrophages following adherence to a fibronectin surface (Thorens et al. 1987). Similarly adherence to a type IX collagen matrix has been shown to induce IL-1 expression by human monocytes (Dayer et al. 1986). Since it has been shown that human osteoblasts have the means to adhere to different substrates, it is important to determine the influence of the matrix on their cytokine expression.

In addition to providing an environment for the bone cells, the extracellular matrix might also determine the response of the bone cells to certain growth factors. For

example, it has been shown that many growth factors, including TGFβ, IGFI, IGFII and FGF are present in the extracellular matrix in a complexed form (Yamaguchi et al 1990; Canalis 1988; Vlodavsky et al 1987). FGF has been shown to bind to certain heparan sulphate proteoglycans and this interaction is thought to be a prerequisite for receptor binding (Yayon et al. 1991). Therefore, since the exogenously added growth factors in this study were not in this complexed form, it is possible that certain effects could not be observed. In the case of TGFβ it has been suggested that this protein is associated with the small extracellular proteoglycan decorin. This association results in a reservoir of TGFβ close to the cell surface. It is possible that this proteoglycan complex is necessary to achieve the correct concentration and conformation of TGFβ required for maximum biological effect. This "juxtacrine' regulation of human bone cell function may be an important factor in modulating cytokine expression.

It is not only the cell : matrix associations that are perturbed when osteoblasts are removed from the bone microenvironment to be cultured. Many cell : cell interactions are also lost, including the direct contact between osteoblasts, and the communication these cells make with osteocytes and bone marrow tissue. Obviously during culture an artificial manner of contact is adopted between the human osteoblast-like cells. It is not unreasonable to expect that this perturbation of cell : cell contact may also affect the expression of many factors, including cytokines.

It is also possible that during the length of time the human osteoblast-like cells are in culture (approximately 6 weeks), their phenotype might not be maintained. In other cell types, including chondrocytes, culture conditions have been shown to promote "de-differentiation" of the cells (Aydelotte, 1988). The reasons for this "de-differentiation" are not totally clear, but are thought to be related to a radical change in environment. Since the tissue culture conditions used were far removed from a natural bone microenvironment, it is important to question whether the cells represent an authentic osteoblast phenotype. The only way this question can be addressed is by comparing the expression profiles of cytokines *in vitro* with those *in situ*..

In situ studies

The results from the *in situ* hybridization studies confirmed that cytokine expression by human osteoblasts is not just a tissue culture artefact. Both TGFβ and IL-1β were shown to be expressed by osteoblasts *in situ*. The expression profiles observed *in situ* shared certain similarities with those seen *in vitro*. This was particularly true for IL-1β expression, where the short lived expression observed *in vitro* mirrored the transient expression seen *in situ*. However there were some differences in the results obtained from the two approaches. Whilst TGFβ expression was constitutive in culture, the expression observed *in situ* was seen to alter during the different stages of osteophyte development. This was not unexpected since the osteoblasts in the bone environment receive many different signals to those in culture. The *in situ* studies did not just confirm the results of the *in vitro* experiments, but served as an extension of the project to characterise cytokine expression during bone remodelling.

The fact that the cytokines were shown to be expressed by distinct cell types at certain stages of osteophyte development is the most compelling evidence reported so far that cytokines have a primary role in the control of local bone remodelling. The importance of this fact cannot be underestimated since, before this study, the impact of cytokine expression on human bone remodelling had not been extensively addressed. The *in situ* studies detected cytokine expression at the mRNA level, no experiments were completed to confirm that functional proteins were present.

The detection of cytokine mRNAs in human bone cells, however, does not necessarily indicate that these factors are active in the local bone environment. There are many mechanisms by which the actions of cytokines may be modified after mRNA synthesis. These different "levels of control" are described in the following sections.

(a) Transcriptional and post-transcriptional modulation.

Studies by Howell et al. (1992) suggest that there is a post-translational regulation of the IL-2 pathway in chronically inflamed rheumatoid synovium. IL-2 mRNA was detected in tissue from 18 needle biopsies by *in situ* hybridization techniques. However immunolocalization studies showed no IL-2 protein could be detected in any

of the samples. The idea that IL-2 expression may be regulated in a post-transcriptional manner is further supported by the observation that isolated synovial lymphocytes fail to produce IL-2, despite the high levels of IL-2 mRNA detected by Northern blotting (Combe et al. 1985; Buchan et al. 1988).

Such factors as the biological half-life of the mRNA may be important for the regulation of cytokine expression. Studies on the newly characterised interleukin, IL-12 suggest that it may contribute to the control of IFNγ expression by T cell blasts (Chan et al. 1992). In conjunction with IL-2, IL-12 treatment has been shown to modulate the IFN-γ mRNA half-life in these cells. T cell blasts were stimulated with IL-2 and IL-12 for 4 hours and then treated with the RNA synthesis inhibitor actinomycin D. Northern blot analysis of the RNA revealed that the IFN-γ mRNA half-life was doubled. IL-2 or IL-12 alone failed to give this mRNA stabilization, highlighting the fact that cytokines may interact together in a complex network.

In the same study IL-12 was also shown to also have an effect on the transcription of the IFN-γ gene. After a 2.5 hour stimulation with IL-12, the transcriptional rate of the IFN-γ gene was increased by 3-fold in the T cell blasts.

(b) Post-translational modification.

Post-translational modifications occur after the synthesis of the cytokine protein. Common modifications include the derivatization of amino acid moieties or glycosylation of the polypeptides. Another modification is the proteolysis of a proform of the cytokine within the cell, to give an active molecule which is subsequently secreted. The activity of IL-1β is controlled in this manner: a 33kDa proform is synthesized which is cleaved into an active 17kDa polypeptide. Recently, the protease which carries out this cleavage has been cloned (Cerretti et al. 1992). This "convertase" is unusual in the fact that it requires proteolytic processing before it is active. This activation step may be catalysed by another enzyme or may be due to an autocatalytic event. This example illustrates how the biological activity of IL-1β may be controlled in several ways, even after it has been synthesized.

(c) Synthesis in a latent rather than active form.

TGFβ has been shown to be expressed by osteoblasts from several species in a latent form (Gehron-Robey 1988). For this cytokine to be biologically active it is necessary for the LAP peptide to be removed by proteolysis or acidification (Lyons et al. 1988). Obviously this extra activation step means that there are additional points at which the action of this cytokine may be modulated. The activity of this cytokine might be restricted to specialized acidic environments (eg under the ruffled border of osteoclasts) where activation may take place. One advantage of the synthesis of a cytokine in a latent form is that the signal is very long lived, using this mechanism it is possible to lay down deposits of cytokines in the matrix for signalling over a long time course.

(d) Presence on cell membrane rather than secretion.

It has been suggested that IL-1α is cell membrane-associated rather than secreted. It is probable that the occurrence of a cytokine on the cell surface of the cell restricts the way in which the cytokine can be used to signal to other cells. In this case it would be necessary for the target cell to make close contact with the signalling cell for the cytokine signal to be mediated. However this mechanism makes the signal highly specific since only the target cell receives the signal. IL-1α is thought to be involved in B cell:T cell signalling using this mechanism (Dinarello, 1989).

(e) Cytokine binding proteins.

Several proteins have been shown to be capable of binding cytokines and growth factors. Binding may cause permanent or temporary inactivation of the ligand as in the case of α_2 macroglobulin cytokine binding (Lamarre et al 1991), or increase cytokine activity (Wang et al. 1991). There are cases where the derivatization of the ligand increases its activity: for example high density lipoprotein (HDL) conjugated LPS stimulates human peripheral blood monocytes to a greater degree than unconjugated LPS.

Recently, several specific high affinity binding proteins have been found for the IGF growth factors. Osteoblasts from several species have been shown to synthesize these binding proteins (IGFBPs) in addition to the IGFs themselves (Ernst and Rodan,

1990). The binding of the IGF to the IGFBP is thought to be reversible and may be a protective mechanism to prohibit premature degradation of these growth factors.

The occurrence of anti-cytokine antibodies in the circulation raises the question as to whether these binding proteins alter the biological activity of cytokines. A study by Bendtzen et al. (1989) demonstrated that specific autoantibodies to IL-1α and TNFα were detectable in the serum of apparently normal adults. The frequency of detectable autoantibodies to IL-1α and TNFα in the population was 24% and 48% respectively. The most prevalent subtype of antibody detected was IgG4. Cytokine-antibody complexes were shown to undergo limited lattice formation and complement activation suggesting that they would be cleared at a low rate. The function of these autoantibodies is not known but it is possible that they may prolong the existence and/or the activity of cytokines in the circulation.

IL-1α was shown to bind avidly to the Fab fragments of these IgG molecules with a dissociation constant of $< 5x10^{-11}M$. It is interesting to note that this avidity is at least an order of magnitude higher than that between IL-1α and other target cells such as fibroblasts and endothelial cells ($kd > 5x10^{-10}$) (Dower et al. 1985). In contrast, lymphoid cells have a greater affinity for this cytokine ($kd < 10^{-12}M$) (Lowenthal et al. 1986). Therefore, IL-1 may be released in an active form to specific target cells containing high affinity binding sites via this mechanism.

(f) Modulation of the receptor.

It does not seem to be the case that cytokine receptors on human osteoblasts are modulated by cytokines. This is in contrast with many other cell types where a cytokine stimulation signal markedly alters cytokine receptor levels, for example with immune cells, whearby IL-1 is a very potent stimulator of IL-2 receptor expression on the surface of T cells (Leonard et al. 1985).

Soluble receptors for several cytokines have been shown to be produced in certain circumstances. The soluble receptors are produced by two mechanisms. The first is the shedding of a fully formed receptor from the cell surface, this has been shown to be the case for TNFα (Seckinger et al. 1990a), IL-2 (Robb et al 1987) and IL-1 (Symons

et al. 1991). Soluble TNFα receptors have been detected in nanogram amounts in the urine of patients with high fever or cancer (Lantz et al. 1990). It is thought that the binding of the ligand to the shed receptor results in a extension of its activity since the receptor complex is difficult to clear. The second mechanism is post-transcriptional, whereby the primary transcript is alternatively spliced to give a shorter transcript coding for a receptor without a transmembrane domain. Such alternative splicing has been demonstrated to take place during the formation of the soluble IL-4 receptor (Mosely et al. 1989)

(g) Interference with receptor binding.

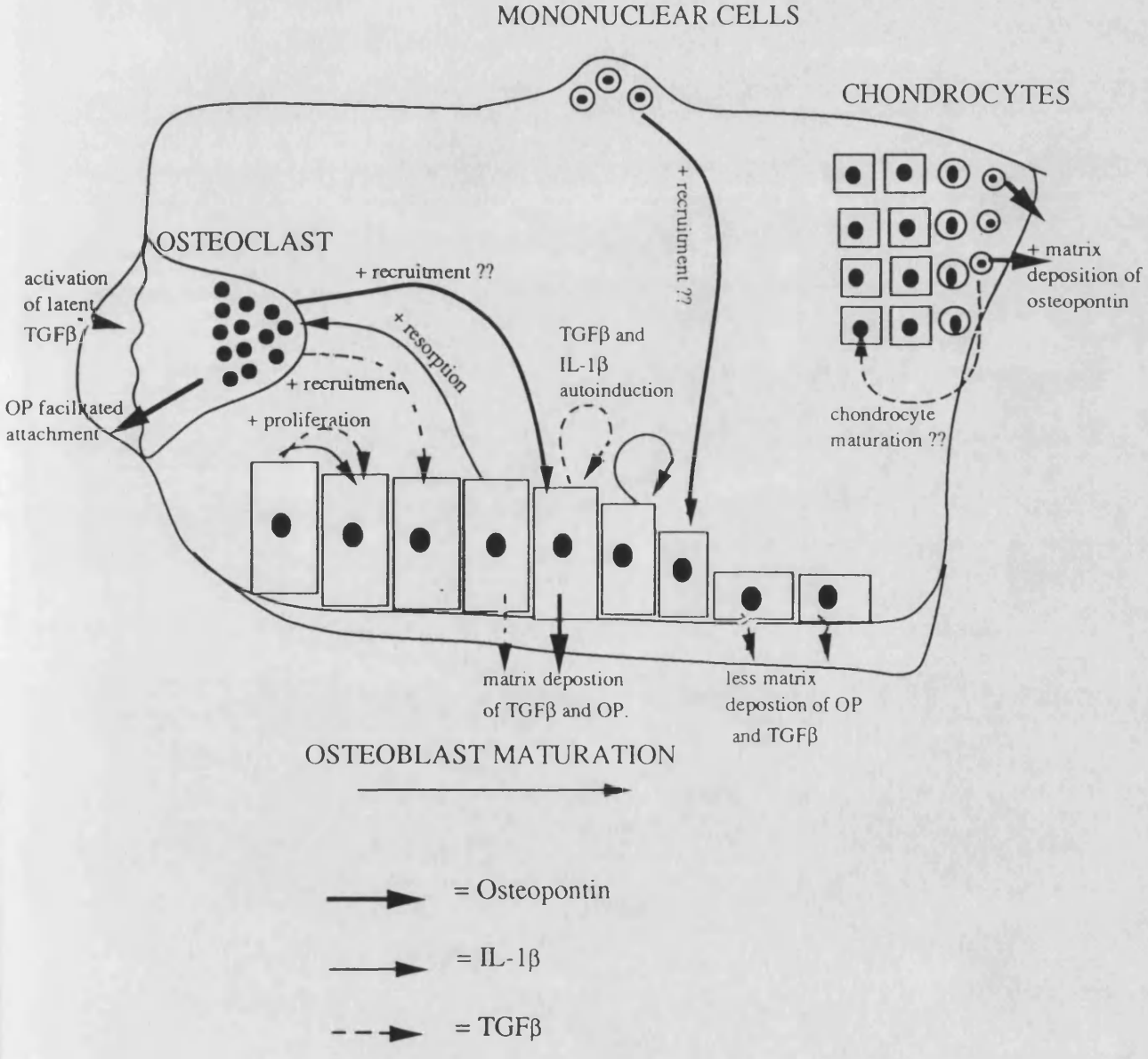
The existence of a naturally occuring IL-1 receptor antagonist (IRAP) suggests that there is yet another control point for cytokine activity. IRAP has been shown to compete with IL-1 for receptor binding, which is thought to result in the abrogation of IL-1 activity. Seckinger et al. (1990b) demonstrated that recombinant IRAP could block IL-1-induced bone resorption and prostaglandin production, in both the fetal rat long bone and rat calvariae models. Interestingly PTH- and TNFα–induced resorption was not blocked, which probably reflects the fact that these effects are independent of an IL-1 receptor signal transduction pathway. Intravenous injection of IRAP produces no symptoms or changes in the parameters associated with IL-1 bioactivity. Healthy human volunteers injected with massive doses of IRAP (10mg/kg) show no significant changes in metabolism. If the equivalent amount of IL-1 was injected the subjects would be very ill (Dinarello and Thompson 1991). Whether there are similar polypeptide receptor antagonists for other cytokines is an important question to be addressed in the future.

The role of osteoclasts in bone remodelling

The results from the *in situ* studies also raise an important question of the role of osteoclasts during bone remodelling. The fact that human osteoclasts have been shown to express osteopontin mRNA questions the purely resorptive nature of this cell type. In addition, human osteoclasts have been shown to be capable of expressing TGFβ and IL-6 mRNA (parallel study with A. Littlewood). This might highlight a means by which osteoclasts can signal to other bone cell types. It has been previously proposed that the communication between osteoclasts and osteoblasts is one way, ie. osteoblasts produce signalling factors that control osteoclastic resorption. However, there is no reason why this signalling mechanism should be unidirectional. Indeed, it is highly probable that a bidirectional means of communication would be preferable. If this was the case osteoclasts could signal to osteoblasts in a variety of instances including after the cessation of resorption. During repair of human bone fracture it is observed that the fracture callus is remodelled in a specific and highly controlled manner. The excess bone formed is resorbed on one side of the bone, and formed on the other. From observing instances like this it seems highly probable that active osteoblast : osteoclast "cross talk" exists. Using *in situ* hybridization and immunolocalization techniques it might be possible to address this question further. Unfortunately at present it is not possible to manipulate human bone to investigate the effect of various factors during remodelling.

In view of the results of this and other studies it is proposed that human bone remodelling is controlled by two main groups of factors, the cytokines and the systemic growth hormones. It is thought that the systemic growth hormones control the overall skeletal development, whilst cytokine production controls local bone remodelling. The two different control mechanisms may be superimposed upon each other. Circulating hormones may act on bone cells either directly or indirectly to modulate the synthesis or effects of cytokines, which in turn may control bone remodelling. An overall scheme for the role of TGFβ, IL-1β and osteopontin in the control of human

bone remodelling is shown in figure VIII.1. It can be seen that the scheme represents a complex network of cytokine expression. However, it only represents a small portion of the overall network that is thought to occur in bone remodelling. Hopefully with the advent of more sophisticated techniques it will be possible to unravel the mechanism of human bone remodelling so that the problems of many bone diseases may be addressed in the future.



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